EPA Reviewer: Linda Taylor, Ph.D. **Signature**: _____
Risk Assessment Branch VII, Health Effects Division (7509P) **Date:** _____
EPA Secondary Reviewer: Elizabeth Mendez, Ph.D. **Signature**: _____
Risk Assessment Branch VII, Health Effects Division (7509P) **Date:** _____

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DATA EVALUATION RECORD

STUDY TYPE: F1-Extended One Generation Toxicity Study – rat
OPPTS 870.3800, 870.6300, 870.7800/OECD 416

PC CODE: 030001 **DP BARCODE:** D376556

TEST MATERIAL (PURITY): 2, 4 D

SYNONYMS: (2,4-Dichlorophenoxy) acetic acid, 2,4-D Acid

CITATION: Marty, MS; Zablotny, CL; Andrus, AK; *et al.* (2010) 2, 4-D: An Extended One Generation Dietary Toxicity Study in Crl:CD(SD) Rats. Toxicology & Environmental Research and Consulting. The Dow Chemical Company. Laboratory Project Study ID 081104, January 30, 2010. MRID 47972101. Unpublished.

Saghir, S. A., Zablotny, C. L. Marty, M. S. Perala, A. W. and Yano, B. L. (2008). A Dietary Dose Range-Finding and Pharmacokinetic Study of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in the Pregnant CRL:CD(SD) Rat and Its Offspring in Preparation for a Subsequent F1-Extended One-Generation Toxicity Study in Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Laboratory Project Study IDs 071153 and 071153A, May 2, 2008. MRID 47417901. Unpublished.

Saghir, S. A., Perala, A. W., and Clark, A. J. (2008). A dietary titration study of 2,4-dichlorophenoxyacetic acid (2,4-D) pharmacokinetics in female CRL:CD(SD) rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Laboratory Project Study ID 071210, April 29, 2008. MRID 47417902. Unpublished.

SPONSOR: Industry Task Force II on 2, 4-D Research Data

EXECUTIVE SUMMARY: In an extended dietary one-generation reproductive toxicity study (MRID 47972101), 2,4-dichloro phenoxyacetic acid (2,4-D; 97.85%-98.6% a.i.; *lot* # 2006 2433 8006-USA) was administered to 27 Crl:CD(SD) young adult rats/sex/dose *via* the diet at dose levels of 0, 100, 300, or 600 (females)/800 (males) ppm [equivalent to 0, ≈5, 15, or 30 (females)/40 (males) mg/kg bw/day] for approximately four weeks prior to mating and continuing through mating (up to 2 weeks), gestation, and lactation. P1 males were exposed for a minimum of 11 weeks including 7 weeks from the initiation of the mating phase. P1 females were exposed until lactation day 22 (LD22). A satellite group of P1 females (12/dose) were subject to the same exposures as the P1

females on the main study (exposure for 4 weeks during the pre-mating, up to 2 weeks during the mating period and during gestation until termination on gestation day 17 (GD 17). Satellite males were not exposed to dietary 2, 4-D except during co-housing with satellite females during the mating period.

P1 Generation: A comprehensive evaluation of P1 male and P1 female reproductive system was conducted, including an evaluation of gonadal function, the estrous cycle, sperm parameters, mating performance, conception, gestation, parturition and lactation, as well as survival, growth and development of the offspring. Selected systemic toxicity parameters were also evaluated in the P1 males and P1 females.

Satellite GD 17 Females: A satellite group of P1 females (12/dose) was included for assessments of selected systemic toxicity parameters, clinical chemistry/hematology, thyroid hormone levels, thyroid weights, plasma 2, 4-D levels, histopathology, and selected reproductive parameters during gestation (corpora lutea and implantation numbers).

F1 Generation: F1 offspring were evaluated for potential effects on the nervous system, immune system, reproductive and endocrine systems, thyroid function, and other systemic toxicity parameters. 2, 4-D plasma levels were also assessed in the F1 offspring. In-life parameters in all F1 offspring included clinical observations, body weights, feed consumption, anogenital distance, nipple retention and puberty onset. Selected F1 offspring were divided into three different groups (Sets 1, 2, and 3) at weaning (postnatal day 21; PND 21). Each set of F1 offspring was maintained on the test diet until PND 60 (Set 1b F1 offspring), ≈PND 70 (Sets 1a and 2a F1 offspring), or ≈PND 90-139 (Sets 2b and 3 F1 offspring).

Set 1a (10/sex/dose): assessment of general systemic and thyroid toxicity, which included clinical chemistry/hematology parameters, thyroid hormone assessment, and urinalysis (males only). Post-mortem evaluations in Set 1a (PND70) included gross pathology, organ weights and histopathology on a wide range of tissues, including thyroids.

Set 1b (10/sex/dose): developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). On PND 60, Set 1b animals were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. A special stain (Luxol Fast Blue) was used to evaluate brain myelination.

Set 2a (10sex/dose): assessment of potential developmental immunotoxicity (DIT): examination of humoral immune function using the sheep red blood cell (SRBC) antibody-forming cell (AFC) assay on PND 70-74.
Set 2b (10/sex/dose): assessment of potential developmental immunotoxicity (DIT): examination of innate cellular immunity using the natural killer cell (NK) assay on PND 87-93.

Set 3 (23-27/sex/dose): assessment of reproductive/endocrine toxicity, which included estrous cycle evaluation and post-mortem evaluations that focused on reproductive organs, sperm assessment, and ovarian follicle counts on PND 139. TK analyses were conducted on Set 3 males and females on PND 63 and 84 to determine plasma 2, 4-D levels.

In addition, selected pups culled on PND 4 were used to assess thyroid hormone levels. Additional

data were gathered from F1 offspring not assigned to Sets 1-3. On PND 22, unselected weanlings were either perfused for examination of neuropathology (12/sex/dose) or euthanized for assessment of systemic toxicity, which included thyroid hormone assessment, organ weights, and post-mortem examinations (gross pathology and histopathology) in 10/sex/dose.

Reproductive and selected data from the F1 generation were used to assess whether a second generation would be produced. None of the criteria were met (Table 1), and a second generation was not assessed in this study.

P1 Adult Rats: There were no treatment-related deaths or clinical signs of toxicity in either sex of P1 adults. Body weights and body-weight gains were comparable among the groups during the pre-mating and mating phases (both sexes) and during gestation and the latter part of lactation (dams). Prior to dietary adjustment of 2, 4-D concentration during the second week of lactation, the 600 ppm dams displayed a decrease in body weight (LD 7; ↓5%) and body-weight gain (LD 1-4; ↓64%), which is consistent with reduced food intake during the first week of lactation. The reduction in food intake can be attributed to the increase in the actual dose (≈65 mg/kg/day) above the targeted level (30 mg/kg/day) during this time. After dietary adjustment, food intake for the 600 ppm dams was above control levels.

There were no apparent treatment-related effects on hematology, differential white blood cell counts, and prothrombin time, and clinical chemistry and urinalysis parameters were comparable among the groups (both sexes). P1 males displayed increased kidney weights (absolute and relative) at 800 ppm, which were accompanied by histopathological findings (degenerative lesion in the proximal convoluted tubules in the outer zone of the medulla) and are consistent with previous findings that the kidney is a target organ. There were no treatment-related findings in the P1 female kidney. Decreased reproductive and accessory sex gland weights were observed at 300 ppm and/or 800 ppm. These changes, however, are related to the concurrent control being outside of the laboratory historical control range. P1 females at 600 ppm displayed increased uterine weights (↑17%, both absolute and relative), although statistical significance was not attained. There were no alterations in estrous cycle pattern in the 600 ppm F1 females compared to the control, and no significant difference in mean estrous cycle length in P1 females at any dose level compared to the control. There were no significant, treatment-related effects on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperm. Male and female mating, conception, fertility, and gestation indices were comparable among the groups, and post-implantation loss was comparable among the groups. Both the time to mating and gestation length were comparable among the groups.

GD 17 Satellite Females: All P1 satellite females survived to scheduled sacrifice, and body weights were comparable among the groups. Hematology and clinical chemistry parameters were comparable among the groups. Reproductive indices and the numbers of corpora lutea and implantations were comparable among the groups. There was a slight increase in resorptions at 600 ppm (0.9 vs 1.5), although there was wide variability (standard deviations exceed the means). There was a slight increase in post-implantation loss at 600 ppm (9.2 vs 5.5). It should be noted that this observation was not corroborated since post-implantation losses in the P1 adults of the definitive study were comparable amongst all dose groups. Both the 100 ppm and 600 ppm females displayed an increase in thyroid weight (↑9%), but there was no dose-response. There were no statistically significant, treatment-related differences in serum T3, T4, or TSH in the GD 17 satellite females. Although the 600 ppm GD 17satellite females displayed the predicted pattern of thyroid hormone

changes (↓ T3 and ↓ T4 with ↑TSH levels) that suggest 2, 4-D exposure may adversely affect thyroid function at doses above the renal saturation clearance, the thyroid effects noted below renal saturation are not considered sufficiently robust to be adverse.

F1 Offspring: There were no treatment-related effects on the numbers of live or dead F1 pups born/litter or on pup survival or sex ratio. Slightly lower body weights were observed in the 600 ppm pups during early lactation, which coincided with the dams decreased food intake LD 1-4 and LD 4-7). Pup body weight (600 ppm) remained lower in the 600 ppm pups (↓6%) during PND 14-21. There was no significant, treatment-related difference in absolute or relative anogenital distance in either sex and no differences in nipple/areolae retention between control and high-dose groups in either sex. F1 males at 800 ppm displayed a 1.6 days delay in preputial separation (well within normal variability), which was accompanied by a very slight reduction in body weight compared to the control (↓2.1 grams; 99% of control). The age at vaginal opening was comparable among the groups of F1 females.

F1 Offspring Thyroid Assessments: PND 4 - There were no statistically-significant differences in serum T3, T4, or TSH in PND 4 culled pups. T4 was reduced to a similar extent in both sexes at the 300 ppm (↓14%-15%) and 600 ppm/800 ppm (↓12%-14%) dose levels, and female PND 4 pups showed an increase in TSH (↑19%) at 600 ppm. **F1 PND 22 Weanlings** - F1 PND 22 males displayed a statistically-significant reduction (↓28%) in T4 at 800 ppm, and F1 PND 22 females displayed a non-statistically significant reduction (↓20%) in T4 at 600 ppm. T3 was reduced in the males at 300 ppm (↓19%) and 800 ppm (↓13%), but there was no dose response. **F1 PND 62-64** - Both sexes displayed increased TSH at 300 ppm (↑26%) and at 800 ppm (males ↑23%))/600 ppm (females ↑24%)), although the increase in males was not dose-related and none of the differences in thyroid hormone levels were statistically significant. T4 was decreased at 800 ppm in males (↓13%). Though these findings suggest that 2, 4-D exposure may adversely affect thyroid function at doses above the renal saturation clearance, the thyroid effects noted below renal saturation are not considered sufficiently robust to be adverse.

F1 Unselected Offspring (PND 22 weanlings): There were no effects on survival of the unselected weanlings used for systemic toxicity (non-perfused). All treated males displayed a decrease in body weight (↓9%-10%) compared to the control males. Decreased adrenal weights were observed in males at 800 ppm (absolute ↓37% and relative ↓29%). The decreases in kidney (↓15%), liver (↓18%), testes (↓15%), and thyroid (↓14%) weights observed in males at 800 ppm were slightly greater than the body-weight deficit of 10%. Organ weights were comparable among the groups of females. There were no significant differences in perfused absolute brain weights, cerebral lengths and widths or cerebellar lengths and widths in perfused F1 PND 22 weanlings of either sex. There were no neuropathological observations attributed to treatment in the perfused F1 PND 22 weanlings, and no treatment-related changes in myelin in either males at 800 ppm or females at 600 ppm.

F1 Offspring Set 1a (PND 70): All Set 1a pups survived to scheduled sacrifice. Males at 800 ppm displayed decreased body weight (↓11%-17%) and body-weight gains (↓11%-25%) throughout the study period, with the magnitude of the reduction lessening with time of exposure. Females displayed comparable body weight/gain among the groups. Platelet counts were reduced in the 800 ppm males but not in the females at any dose level. Both sexes displayed a slight increase in ALT (↑18%/25%) and an increase in triglyceride (↑31%/43%) levels. Although some of the decreases in organ weights observed in the 800 ppm males may be attributed to the 10% decrease in body weight

at termination, the decreases in liver (↓16%), pituitary (↓14%), and adrenal glands (↓12%) might be related to treatment. Increased uterine weights (↑31% absolute and ↑32% relative) were observed at 600 ppm. Although statistical significance was not attained, the finding is considered treatment-related since a similar increase was observed at 600 ppm in the P1 and Set 3 F1 females. Increased ovarian weight (↑9%) was observed in the 600 ppm F1 Set 1a females, although statistical significance was not attained. Increased kidney weights (↑9% absolute and ↑11% relative) were observed in the females at 300 ppm and 600 ppm, although there was no dose-response and kidney weights were comparable among the male groups. Decreased thymus weights (↓12% absolute and ↓10% relative) were observed in females at 600 ppm and in Set 3 females at 600 ppm (↓14% absolute and ↓13% relative). An increased incidence of degeneration of the proximal convoluted tubule in the kidney was observed in males at 300 ppm and 800 ppm and in females at 600 ppm. Regarding the terminal stage of estrous, 2 of 10 females at 300 ppm and 3 of 10 females at 600 ppm displayed proestrus, whereas none of the 10 females in the control and 100 ppm groups displayed proestrus.

F1 Offspring Set 1b (PND 54-56): There were no significant differences in body weight/gain in either sex. There was an increase in the level of urination in all treated male groups compared to the control group, but there was no dose response. There was a 10% reduction in hind limb grip strength at 800 ppm in males and at 600 ppm in females. Males at 800 ppm displayed a decrease in total motor activity (↓10%), whereas females at 600 ppm showed an increase (↑12%). During the first half of the session, all male groups displayed a similar motor activity level (were within 6%), whereas the 800 ppm males showed a progressive lessening of activity with increased time; *i. e.,* the 800 ppm males displayed decreased activity compared to the control (↓11%, ↓16%, ↓30%, and ↓34% in Epochs 5, 6, 7, and 8, respectively). Males at 800 ppm displayed a different acoustic startle response (ASR) initially compared to the control males. There was no apparent difference in ASR in females. There were no significant differences in perfused absolute brain weights, cerebral lengths and widths, or cerebellar lengths and widths in either sex (PND 60). There were no treatment-related (1) microscopic changes in the central or peripheral nervous system in the perfused offspring; (2) changes in myelin; or (3) changes in microscopic measurements of structures in the cerebral cortex, cerebellum, thalamus, or hippocampus.

F1 Offspring Set 2a (PND 67-73): Developmental Immunotoxicity (Primary Immune Response to Sheep Red Blood Cells): There were no deaths. Slight decreases in body weights and body-weight gains were observed in males at 800 ppm (↓6%-10% and ↓15%) and females at 600 ppm (↓8%-9% and ↓10%). Terminal body weights were comparable among the male and female groups. Both absolute (↓10%) and relative (↓8%) thymus weight decreases were observed in the males at 800 ppm and in the females at 600 ppm [absolute (↓13%) and relative (↓10%)]. Males at 300 ppm showed a 17% decrease in thymus weight but no dose response. Spleen weights were slightly lower in females at 600 ppm [absolute (↓13%) and relative (↓14%)]. There was no significant difference in response for AFC/spleen and AFC/10^6 splenocytes among the male groups. Females at 600 ppm displayed a non-significant decrease of 54% for AFC/spleen and 27% for the AFC/10^6 splenocytes.

F1 Offspring Set 2b (PND 67-73): Developmental Immunotoxicity (Natural Killer Cell Activity): There were no deaths, and body weights/gains showed a similar slight reduction in males at 800 ppm as observed in the other offspring groups. Female body weights/gains were comparable among the groups. Terminal body weights (PND 87-93) were comparable among the groups (both sexes). There were no significant treatment-related effects on absolute or relative spleen or testes weights in males, and no significant treatment-related effects on spleen weights in females (only organs weighed).

There were no significant, treatment-related differences in the percent target cell cytotoxicity at any dose level compared to control (both sexes), and 2, 4-D did not alter the cytotoxic ability of splenic NK-cells in male or female rats at any dose level.

F1 Offspring Set 3 (PND 90 or 139): Reproductive Toxicity: There were no treatment-related deaths or clinical signs of toxicity. Terminal body weights were comparable among the groups (both sexes). No significant differences were observed in mean estrous cycle length at any dose level compared to the control. There were no significant, treatment-related effects on the numbers of small follicles, growing follicles, or total follicles. There were no significant, treatment-related effects on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperm between the control and 800 ppm males. Absolute (↓9%) and relative (↓8%) pituitary gland weights were significantly lower in the 800 ppm males and absolute (↓9%) and relative (↓10%) pituitary gland weights were non-significantly lower in the 600 ppm females. There was no associated histopathology in the pituitary glands. Uterine weights were increased at 300 ppm (↑10% absolute and ↑10% relative) and 600 ppm (↑10% absolute and ↑11% relative) compared to the controls. Thymus weights were decreased (↓14% absolute and ↓13% relative) in females at 600 ppm, although statistical significance was not attained. No histopathological changes were observed in the pituitary or thymus in either sex. A degenerative lesion was observed in the kidney (proximal convoluted tubule) in both sexes at 300 ppm and at 600 ppm/800 ppm. Ovarian follicle counts were comparable between the control and 600 ppm females (PND 139).

The parental systemic LOAEL is 800 ppm (45.3 mg/kg bw/day in males), based on nephrotoxicity manifested as increased kidney weights, and degenerative lesions in the proximal convoluted tubules in the main study P1 rats. The parental systemic NOAEL is 300 ppm (16.6 mg/kg bw/day in males). No toxicologically relevant effects were identified in P1 females or in the GD 17 satellite female groups at the highest dose tested (600 ppm; 40.2 mg/kg/day).

The thyroid toxicity NOAEL is established at 800/600 ppm (45.3 mg/kg/day in males and /40.2 mg/kg/day in females), the highest dose tested. The thyroid effects noted in the database were considered to be adaptive.

The offspring LOAEL is 300 ppm (20.9/ mg/kg bw/day in males and 23.3 mg/kg/day in females), based on kidney toxicity manifested as increased kidney weights and increased incidence of degeneration of the proximal convoluted tubules. The offspring NOAEL is 100 ppm (6.83 mg/kg bw/day in males and 7.59 mg/kg/day in females).

The DNT offspring (PND 21-60) LOAEL is >800/600 ppm (81.7 mg/kg bw/day in males, 59.2 mg/kg bw/day in females), based on the lack of evidence of DNT (FOB parameters, motor activity, and acoustic startle response). The DNT offspring NOAEL is 800 ppm/600 ppm (81.7 mg/kg bw/day in males, 59.2 mg/kg bw/day in females).

The DIT offspring (PND 139) LOAEL is >800/600 ppm (71.8 mg/kg bw/day in males, 55.3 mg/kg bw/day in females), based on the lack of evidence of DIT [SRBC antibody-forming cell assay (PND 66-70) and Natural Killer Cell assay (PND 87-93)]. The DIT offspring NOAEL is 800/600 ppm (71.8 mg/kg bw/day in males and 55.3 mg/kg bw/day in females), the highest dose tested.

The reproductive LOAEL is > 800/600 ppm (45.3 mg/kg bw/day in males, 40.2 mg/kg bw/day in females), based on the lack of effect on estrous cyclicity, (P1 females, satellite GD 17 dams, Set 3 F1 offspring) or reproductive indices (mating, fertility, time to mating, gestation length, pre-and post-implantation loss, number of corpora lutea (satellite GD 17 dams), sperm parameters, ovarian follicle counts, and reproductive organ histopathology). The reproductive NOAEL is 800/600 ppm (45.3 mg/kg bw/day in males, 40.2 mg/kg bw/day in females), the highest dose tested.

This study is classified acceptable/non-guideline. The study does not satisfy a guideline requirement for 2, 4-D. It satisfies the data call-in requirements for 2, 4-D for OPPTS 870.3800 (Reproduction and Fertility Effects), OPPTS 870.6300 (Developmental Neurotoxicity), OPPTS 870.7800 (Immunotoxicity). The study is in accordance with the draft OECD extended one-generation reproductive toxicity study guideline.

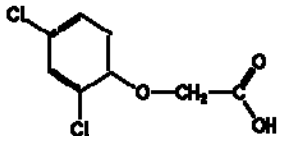
COMPLIANCE: Signed and dated Good Laboratory Practices (GLP), Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test material: 2,4-D

Description:	Solid, off-white flakes (prior to milling)
Lot #:	2006 2433 8006-USA
Purity:	97.85%-98.6% a.i.
Compound stability:	Stable in diet for 27 days
CAS # of TGAI:	94-75-7
Structure:	



2. Vehicle and/or positive control: none (test material was milled and mixed with certified feed flour, then diluted with ground feed).

3. Test animals:

Species:	Rat
Strain:	Crl:CD(SD)
Age at study initiation:	(P) 10 wks; (F_1) wks
Wt. at study initiation:	(P) Males: 340-345 g; Females: 231-233 g (F_1) Males: g; Females: g
Source:	Charles River Laboratories Inc. (Portage, Michigan)
Housing:	Singly in stainless steel cages, except during mating (1 ♂/1 ♀), littering, early post-weaning phases of study; during littering, dams (and their litters) were housed in plastic cages provided with ground corn cob nesting material from ≈GD 19 until PND 21.
Diet:	LabDiet Certified Rodent Diet #5002, *ad libitum* (PMI Nutrition International, MO)
Water:	Municipal water, *ad libitum*
Environmental conditions:	**Temperature:** 22 ± 1°C **Humidity:** 40-70% **Air changes:** 12-15/hr **Photoperiod:** 6 hrs dark/6 hrs light
Acclimation period:	≥ one week

B. PROCEDURES AND STUDY DESIGN

1. Mating Procedure: In the main study, each P1 female was placed with a single P1 male from the same dose level (1:1 mating). High-dose males had access to the female high-dose diet during this period to avoid overdosing the high-dose females. In the satellite groups, each female was placed with an unexposed male that had access to a 2,4-D-containing feed crock during the mating period only. The rats were paired until mating occurred or two weeks elapsed. During the mating period, daily vaginal lavage samples were evaluated for the presence of sperm as an indication of mating. The day on which sperm were detected or a vaginal copulatory plug was observed *in situ* was considered gestation day 0 (GD 0). Each sperm- or plug-positive (presumed pregnant) female was then separated from the male and returned to her home cage. If mating had not occurred after two weeks, the rats were separated without further opportunity for mating. After the mating phase, satellite males were euthanized with no further data collection.

2. **Study Schedule:** Mating of the P1 adults (main study animals and females from a satellite group) commenced after approximately 4 weeks of dietary exposure to 2,4-D. P1 males from the satellite group were not exposed to 2,4-D except during the mating period. Main study P1 male rats were maintained on the test diets through breeding and for an additional 5-7 weeks (11 weeks total exposure). P1 females (main study and satellite females) continued on the test diets through breeding, gestation (satellite females through GD 17 only), and lactation (study report Figures 1 and 2, reproduced below). Triggers for breeding a second generation were identified prior to study initiation (Table 1). Since these criteria were not met, breeding of a second generation was not conducted.

Table 1. Triggers for Decision Making on the Need for a Second Generation	
P1 Estrous Cycle Evaluation	Trigger[1]
P1 Fertility	Trigger[2]
F1 Litter Parameters	Trigger[3]
F1 Developmental Landmarks (AGD, nipple retention, puberty onset)	Trigger[4]
F1 Estrous Cycle Evaluation	Trigger[1]
1 If biologically relevant, dose-related changes in estrous cycle length without overt toxicity in dams 2 In the absence of corresponding, exposure-related reproductive organ histopathology 3 If significant or biologically relevant, exposure-related decreases in litter size/pup survival are seen in the absence of severe maternal toxicity or lethality 4 Dose-related effects; in the absence of body weight-mediated changes in these parameters	

3. **Animal Assignment:** Prior to dose initiation, rats were stratified by body weight and randomly assigned to exposure groups (Table 2) using a computer program designed to increase the probability of uniform group mean body weights and standard deviations at the start of the study.

TABLE 2. Animal Assignment (Study Phases)			
Dose (ppm)	**MALES**	**Dose (ppm)**	**FEMALES**
0 100 300 800	P1 males (main study; n=27)	0 100 300 600	P1 females (main study; n=27) **B**
	P1 males (satellite; n=12) **A**		P1 females (satellite; n=12)
	F1 Set 1a (systemic toxicity; n=10) **C**		F1 Set 1a (systemic toxicity; n=10) **D**
	F1 Set 1b (developmental neurotoxicity; n=10) **E**		F1 Set 1b (developmental neurotoxicity; n=10) **F**
	F1 Set 2a (developmental immunotoxicity; SRBC; n=10) **E**		F1 Set 2a (developmental immunotoxicity; SRBC; n=10) **F**
	F1 Set 2b (developmental immunotoxicity; NK cell assay; n=10) **E**		F1 Set 2b (developmental immunotoxicity; NK cell assay; n=10) **F**
	F1 Set 3 (reproductive toxicity; n=24-27) **E**		F1 Set 3 (reproductive toxicity; n=24-27) **F**

***** **A** no data collected since these rats were not treated with 2, 4-D except during mating; **B** On LD 7-14 the dietary concentrations adjusted to 0, 50, 150, and 300 ppm; on LD 14-21 to 0, 33, 100, and 200 ppm.; **C** On PND 21-28 and PND 28-35 the dietary concentrations were adjusted to 0, 50, 150, and 300 ppm and 0, 50, 150, and 400 ppm, respectively. **D** On PND 21-28 and PND 28-35 the dietary concentrations were adjusted to 0, 50, 150, and 300 ppm; **E** On PND 21-28 and PND 28-35 the dietary concentrations were adjusted to 0, 50, 150, and 300 ppm, and 0, 50, 150, and 400 ppm, respectively; **F** On PND 21-28 and PND 28-35 the dietary concentrations were adjusted to 0, 50, 150, and 300 ppm.

2,4-D: AN F1-EXTENDED ONE GENERATION DIETARY TOXICITY STUDY IN CRL:CD(SD) RATS

FIGURE 1. Study Design for the Extended One-Generation Toxicity Study

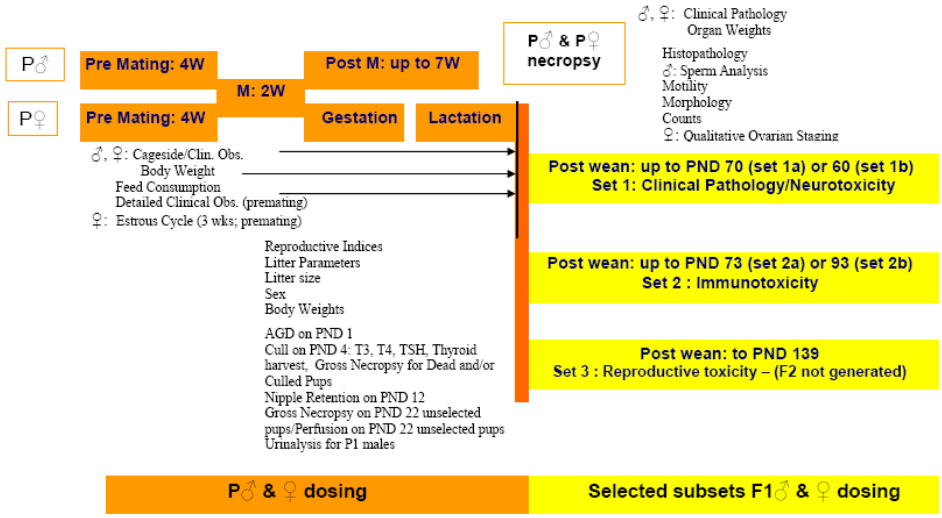
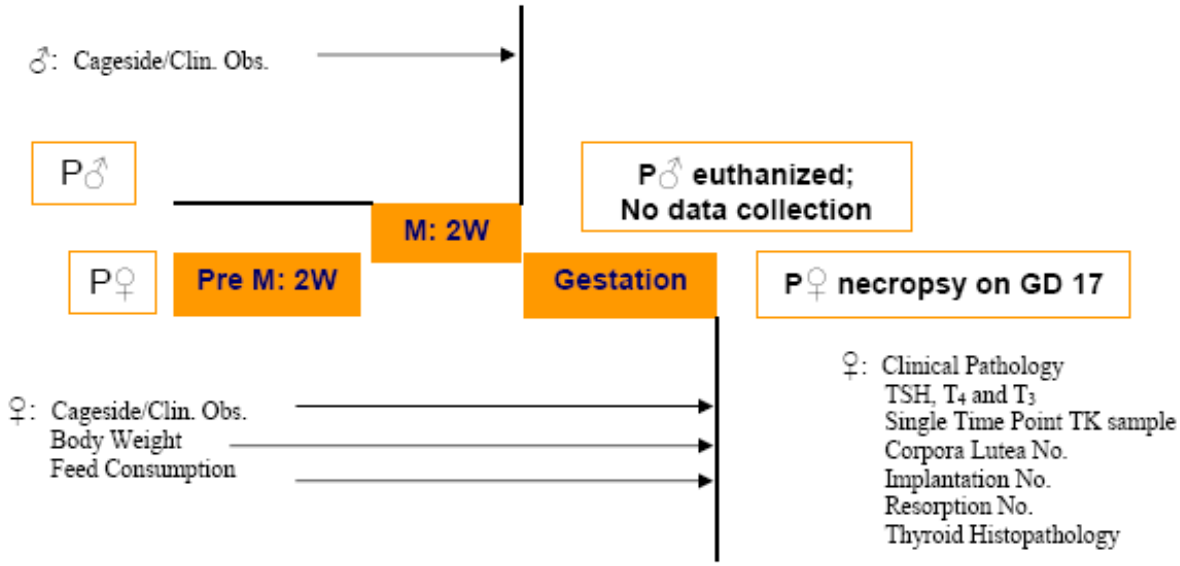


FIGURE 2. Study Design for the Satellite Group in the Extended One-Generation Toxicity Study



4. Dose Selection Rationale: Doses were selected (Table 3) based on a combined analysis of toxicity and toxicokinetic (TK) data collected from the 2,4-D range-finding/TK study (APPENDIX A - MRID 47417901; abbreviated DER) and titration studies (APPENDIX B - MRID 47417902; abbreviated DER). The high dose was set at or slightly above the threshold for nonlinear kinetics. As stated in the report, the range-finding study confirmed a gender-based difference in the renal clearance of 2,4-D in adult rats, and different high-dose levels were selected for the sexes. The male high dose was 40 mg/kg/day (dietary concentration of 800 ppm), slightly higher than the inflection point for nonlinear TK in male pups from PND 35 to adulthood. The female high dose was 30 mg/kg/day (600 ppm), which was clearly higher than the inflection point in female pups and adults throughout the entire (range-finding) study. The mid- and low-dose levels were the same for both sexes. The low dose of 5 mg/kg/day (100 ppm) was predicted to identify a clear NOAEL and was consistent with the dose identified in a previously conducted 2,4-D dietary 2- generation reproduction study (MRID 00150557, MRID 00163996). The mid-dose level of 15 mg/kg/day (300 ppm) would provide dose-response data. It was anticipated that the 300 ppm mid-dose level would be slightly above the inflection point for non-linear TK in female adults and pups, and within the range of linear TK for adult males. The dose range-finding and toxicokinetic studies conducted in pregnant rats indicated that 2,4-D was adequately transferred through maternal milk to pups (MRIDs 47417901 and 47417902). The 2,4-D pup/dam plasma ratios on LD 4 were 0.29, 0.30, and 0.48 for dams treated with 100, 400 and 800 ppm 2,4-D, respectively. By LD 14, the pup/dam plasma 2,4-D ratios increased to 0.75, 1.25 and 1.70 for the respective dietary doses. Because there was evidence of significant transfer of 2,4-D to neonatal pups through milk (µg 2,4-D/g milk on LD 4 were: 1.08 at 100 ppm, 9.27 at 400 ppm and 30.84 at 800 ppm), it was not necessary to directly gavage dose neonates with 2,4-D.

Table 3. Study Design.

Dietary Concentration (ppm)		≈ Doses (mg/kg/day)		P1 #rats/sex/dose	Satellite group (targeted # pregnant females/dose)
male	female	Male	female		
0	0	0	0	27	12
100	100	5	5	27	12
300	300	15	15	27	12
800	600	40	30	27	12

5. Dietary Concentration Adjustment: To avoid potential overdosing of females during mating, co-housed rats were fed the female diet. No dietary concentration adjustments were performed during gestation or during the first week of lactation. The dietary concentration adjustments during lactation and post-weaning were designed to provide a relatively constant mg/kg body weight/day dose throughout all life phases, these are outlined in Table 4.

Table 4. 2,4-D Dietary Concentration Adjustments During Lactation/Post-Weaning

Exposure period	TMI Increase[1]	Unadjusted (ppm)	Adjustment Factor	Adjusted (ppm)
LD 7-14	**3.1X**	**100, 300, 600**	**2X**	**50, 150, 300**
LD 14-21	**3.8X**	**100, 300, 600**	**3X**	**33, 100, 200**
PND 21-28	**2.4X**	**100, 300, 600**	**2X**	**50, 150, 300**
PND 28-35	**1.9X**	**F: 100, 300, 600 M: 100, 300, 800**	**2X**	**F: 50, 150, 300 M: 50, 150, 400**

[1]relative to non-pregnant adult females; TMI= test material intake based on feed consumption data derived from male Crl:CD(SD) rats at PND 23-28 from Marty *et al.*, 2003.

During lactation, dietary concentrations were adjusted using historical control feed consumption data for lactating females to account for the large and rapid increase in feed consumption (2-3-

fold) typical for dams in mid to late lactation. Dams awaiting necropsy received a diet containing the same concentration of 2, 4-D that was given during breeding. To account for the large amount of feed consumed per kg body weight in weanling pups from PND 21-28, male and female weanlings received a diet containing the same concentration of 2, 4-D that was given to the P1 females during the second week of lactation (one-half the adult female concentration). From PND 28-35, female weanlings received a diet containing the same concentration of 2, 4-D that was given to the P1 females during the second week of lactation (one-half the adult female concentration). Male weanlings received a diet containing one-half the adult male concentration of 2, 4-D during PND 28-35; i.e., concentrations in the mid- and low-dose groups for male weanlings during this interval paralleled that of the female weanlings, but the high-dose male weanlings received a higher dose than female weanlings of the same age. F1 male and female offspring were transitioned to adult dietary concentrations of 2, 4-D on PND 35, which continued until termination.

6. **Dosage Preparation and Analysis**: Prior to diet mixing, the test material was air milled (particle size: ≈149 micron) and used for preparation of a concentrated test material-feed mixture (pre-mix). Each pre-mix was prepared by mixing test material with certified feed flour to minimize clumping, then diluting this mixture with ground feed to achieve the targeted pre-mix concentration. Diets were prepared by diluting pre-mixes with ground feed. Control diets were prepared by mixing feed flour with ground diet using a similar procedure. Pre-mixes and test diets were not adjusted for purity and were prepared every 3 weeks based on stability data.

   Analysis: Homogeneity: Representative samples of the low- and high-dose test diets were evaluated concurrently with the concentration verification analyses to ensure homogeneous distribution of the test material in the feed.

   Stability: In the range-finding study (MRID 47417901), stability of 2, 4-D in the diet was determined to be 27 days. Fresh diets were prepared at least once every 3 weeks for the main study. Additional stability work was conducted to verify that the lowest concentration in the main study was stable for the 3-week feeding period.

   Concentration Verification: Dose confirmation analyses of all dose levels, plus control and premix, were determined pre-exposure. Dose confirmation included sampling during the following study phases: 4 weeks (end of pre-breeding period), 8 weeks (approximately the end of gestation), 12 weeks (at weaning), 15 weeks, and 19 weeks. The homogeneity of the low-dose and high-dose diets was determined concurrent with dose confirmation. The method used for analyzing the test material in the diet was a solvent extraction followed by analysis using liquid chromatography-mass spectrometry (LCMS) with internal standards.

   Results: Homogeneity analysis: Analyses of low-dose and high-dose diets indicated that 2, 4-D was homogenously distributed in the feed based on the mean and relative standard deviations, ranging from 2.6 to 11.2%. **Stability analysis:** Previously, 2, 4-D was shown to be stable in the diet for < 27 days at concentrations ranging from 0.01 to 0.5% (Marty and Andrus, 2007). Additional stability work verified that lower dietary concentrations used during the study (0.0033%) were stable. Diets were used within 3 weeks of preparation. **Concentration analysis:** Analyses of the test diets for concentration verification were conducted on nine different occasions during the study. The average concentrations of 2, 4-D in the diets were 86.7% to 106.8% of the targeted concentrations. Of the 55 samples analyzed over the course of the study,

all but four samples were within + 10% of nominal and all analyses were within + 15%.

C. METHODS

PARENTAL ANIMALS

1. **Observations:** Observations and the schedule for those observations are shown in Figures 1 and 2 (reproduced from study report pages 190-191).

   a. **Cageside Observations:** All rats were observed for morbidity and mortality at least twice daily. Cageside examinations (rats not hand-held) included an assessment for decreased/increased activity, repetitive behavior, vocalization, in-coordination/limping, injury, neuromuscular function (convulsion, fasciculation, tremor, and twitches), altered respiration, blue/pale skin and mucous membranes, severe eye injury (rupture), alterations in fecal consistency, and fecal/urinary quantity.

   b. **Clinical Examinations:** Clinical observations were conducted on all males prior to exposure and weekly thereafter; on all females pre-exposure and weekly throughout the pre-breeding and breeding periods. Mated (sperm-positive or plug-positive) females received clinical examinations on GD 0, 7, 14, and 20. Females that delivered litters were subsequently evaluated on LD 0, 1, 4, 7, 14, and 21. Females were observed for signs of parturition (≈GD 20), and dams were examined for signs of abnormal nursing behavior. Examinations included a careful, hand-held examination of the rat with an evaluation of abnormalities in the eyes, urine, feces, gastrointestinal tract, extremities, movement, posture, reproductive system, respiration, skin/hair-coat, and mucous membranes, as well as an assessment of general behavior, injuries, or palpable mass/swellings.

   c. **Detailed clinical observations (DCO):** The DCO evaluations were performed on the P1 rats of both sexes pre-exposure and once during the last week of the pre-mating period and included cage-side, hand-held, and open-field observations (Table 5).

Table 5. DCO Parameters
Cage-Side Observations
Abnormal movements or behaviors
Resistance to removal from cage

Hand-Held Observations
Eye observations
- Palpebral closure
- Pupil Size
- Lacrimation (non-colored periocular wetness)
Salivation (non-colored perioral wetness)
Muscle tone
Extensor-thrust response
Reactivity to stimuli
Abnormal behavior
Abnormalities of the eye Description
Abnormal urine or feces Description
Abnormalities of the gastrointestinal (GI) tract
Injury
Missing extremity
Abnormal muscle movements
Palpable mass/swellings
Abnormal posture
Abnormalities of the reproductive system
Abnormal respiration
Abnormal skin or hair-coat/mucous membranes
Excessive soiling
General abnormalities
Open-Field Observations
Responsiveness to touch
Gait evaluation

From Table 1, page 227 of the report

d. Dam observations: Dams were observed periodically for signs of parturition beginning on or about GD 20. When possible, parturition was observed for signs of difficulty or unusual duration. The day of parturition was recorded as the day one or more delivered fetuses were noted (designated lactation day 0; LD 0).

2. **Body weight:** All parental rats were weighed during the pre-exposure period and weekly during the 4-week pre-mating period. Males were weighed weekly after mating until termination. Mated females were weighed on GD 0, 7, 14, and 15 (satellite group)/20 (main study). Lactating females were weighed on LD 1, 4, 7, 14, and 21.

3. **Food consumption and compound intake:** Food consumption was measured at intervals similar to body-weight measurements. For females delivering litters, food consumption was measured every 2-4 days.

4. **Estrous Cycle Evaluation:** Vaginal lavage samples from all P1 females were collected daily beginning one week after the initiation of dosing. Estrous cycles were evaluated for 3 weeks prior to mating, and during cohabitation until each female was sperm-or plug-positive or until the two week mating period had elapsed. Vaginal lavage slides collected during the 3-week period prior to mating were examined microscopically to determine estrous cycle length and pattern. On the day of scheduled necropsy, the stage of the estrous cycle was also determined.

5. **Sperm Analyses:** Sperm parameters were evaluated in P1 and Set 3 F1 males at termination. The left and right epididymides and testes were allocated as follows: right epididymis: motility and histopathology; left epididymis: counts; right testis: histopathology; left; testis: counts.

a. Motility: Sperm motility was evaluated in P1 males from all dose groups. Immediately after euthanasia and isolation of their epididymides, a small sample of sperm from the right cauda epididymis was processed (placed in chamber of the HTM Integrated Visual Optical System) for the determination of total percent motile (showing any motion) and percent progressively motile (showing net forward motion) sperm. After sperm were released, the epididymis was placed in Bouin's fixative for histopathological examination.

b. **Counts**: The left testis and cauda epididymis were weighed and then frozen at ≈ -20°C for subsequent determination of the number of homogenization-resistant spermatids and sperm per testis/cauda epididymis and per gram of testicular/epididymal tissue. Spermatid/sperm counts were evaluated from the control and high-dose groups, as well as any males that failed to mate successfully during the mating period. Since treatment–related effects were not observed, sperm from the lower-dose levels were not evaluated.

c. **Morphology**: An aliquot of sperm suspension was placed on a slide, and a smear prepared and air-dried for subsequent evaluation of sperm morphology. At least 200 sperm per male were evaluated and classified as normal or abnormal. Morphological evaluation of sperm was conducted using samples from the control and high-dose groups and any male that failed to mate successfully during the mating period. Sperm morphology was scored "blinded" with respect to exposure group. Since treatment-related effects were not observed, sperm from the lower-dose levels were not evaluated.

OFFSPRING

1. **Litter Observations:** Litters were examined as soon as possible after delivery (Table 6). Dead pups were sexed, examined grossly for external and internal abnormalities, and preserved. Any visible physical abnormalities or demeanor changes in the neonates were recorded as they were observed during the lactation period on LD 0, 1, 4, 7, 14 and 21. Any pup found dead or sacrificed in moribund condition was sexed and examined grossly, to the extent possible, for external and visceral defects. These pups were preserved in neutral, phosphate-buffered 10% formalin.

TABLE 6. Litter Observations [a]

Observation	Time of observation (lactation day)					
	Day 0	Day 1	Day 4	Day 7	Day 14	Day 21
Number of live and dead pups	X	X	X	X	X	X
Pup sex and body weight		X	X [b, c]	X	X	X
External alterations or demeanor changes	X	X	X	X	X	X

[a] Data obtained from page 57 in the study report. [b] Before standardization (culling). [c] After standardization (culling)

Pups that died or appeared moribund were examined for the presence of milk bands (i.e., milk in the pup's stomach that was visible on external examination). F1 offspring received weekly clinical observations until necropsy. Examinations included a careful, hand-held examination of the rat with an evaluation of abnormalities in the eyes, urine, feces, gastrointestinal tract, extremities, movement, posture, reproductive system, respiration, skin/hair-coat, and mucous membranes, as well as an assessment of general behavior, injuries, or palpable mass/swellings.

2. **Body Weight:** The F1 offspring were weighed weekly beginning on PND 21, on the day of

puberty onset (*i.e.,* vaginal patency or preputial separation), and at termination. The Set 1b F1 offspring also were weighed on the day of the FOB and acoustic startle measurement.

3. **Food Consumption:** Feed consumption was determined weekly beginning when the F1 offspring were individually housed on PND 28 until the day prior to necropsy. Feed consumption was calculated as above for adults.

4. **Anogenital Distance:** Anogenital distance (absolute and relative to the cube root of body weight) was measured in all F1 pups on PND 1.

5. **Culling and Culled Pups:** All litters were standardized to ten pups per litter on PND 4 by randomly ordering the pups in each litter by sex. Pups culled were randomly selected using a computer generated randomization procedure, so that five males and five females remained in each litter (where possible), or unequal numbers were retained; e.g., six males, four females. Litters with fewer than ten pups were not culled. Preferential culling of runts was not performed. Culled pups that were not used for thyroid sampling (see below) were euthanized, examined grossly for external and visceral defects, and discarded.

6. **F1 Culled Pup Sampling for Thyroid Analysis:** PND 4 culled pups were anesthetized, examined grossly, and blood was collected (heart). To ensure a sufficient volume for hormone analyses, blood was pooled by sex for each litter until a total of 10 samples/sex/dose were obtained. Thyroid glands were harvested from the same subset of the pups used for thyroid hormone analyses. The trachea with the thyroid gland were removed and preserved for possible histopathological examination. Following thyroid gland removal, the animals were euthanized *via* decapitation.

7. **Nipple/Areolae Retention:** All offspring were evaluated for the presence of nipple/areolae on PND 12. The average number of nipples/areolae in male and female offspring in each litter was determined. The mean number of nipples/areolae for males and females in each dose group was calculated from these litter means. Observers were blind to exposure group. Since there was no significant increase in retained nipples/areolae in males on PND 12, Set 3 males were not examined for this endpoint at necropsy.

8. **Weaning and Set Assignment:** All litters were weaned on PND 21. Three male and three female F1 pups/litter were randomly selected, and one male and one female each were assigned to Set 1, 2, or 3. For **Set 1** (**Figure 4;** reproduced from study report page 193), one male or one female per litter were assigned to **Set 1b: the Developmental Neurotoxicity group** (n = 10 males + 10 females/dose level with 20 litters represented). Unselected F1 males or F1 females from each litter designated for Set 1 were assigned to **Set 1a: the Systemic Toxicity group** (n = 10 males + 10 females/dose level with 20 litters represented). Similarly, 20 F1 males and 20 F1 females/dose level were assigned to **Set 2 (Figure 5;** reproduced from study report page 194**): the immunotoxicity group**. One male and one female per litter were assigned to **Set 2a for the initial evaluation of immunotoxicity** with the SRBC AFC assay (n = 10 males + 10 females/dose level with 10 litters represented). One male and one female from the remaining litters designated for Set 2 were used for **Set 2b** (n = 10 males + 10 females/dose level with 10 litters represented). This group was used for a **secondary evaluation of immunotoxicity** (*i.e.*, natural killer (NK) cell assay). One male and one female per litter were randomly selected for assignment to **Set 3 (Figure 6;** reproduced from study report page 195**): the reproductive**

endpoints group (n > 20 males and 20 females/dose level). If there were insufficient litters from which to select both male and female offspring for groups 1, 2, and 3, additional animals were randomly selected from available litters as needed in order to obtain the required number of animals/dose level. Use of same sex littermates in the same Set (1, 2, 3) was avoided whenever possible. The assignment of pups from each litter is shown in study report Appendix Table 161. Pups were transpondered on ~ PND 17 for individual identification after weaning. Selected F1 pups were housed in plastic cages with same sex littermates from PND 21-28, then individually thereafter. **Non-selected pups** (**Figure 1** above and **Figure 3 below**)**:** All non-selected F1 weanlings (designated for pathological evaluation as described below) were given a gross necropsy on PND 22. When possible, one pup/sex/litter (n = 12/sex/dose) at weaning was **perfused for neuropathology evaluations**. Additionally, one pup/sex/litter (n = 10/sex/dose) was randomly selected for a necropsy examination with collection of blood for **thyroid hormone analyses, organ weights**, and preservation of tissues for subsequent histopathological examination. Details of weanling necropsies are described below. Any excess weanlings were discarded.

FIGURE 4. Study Design for F1 Generation Set 1a (Clinical Pathology Group) and 1b (Neurotoxicity Group)

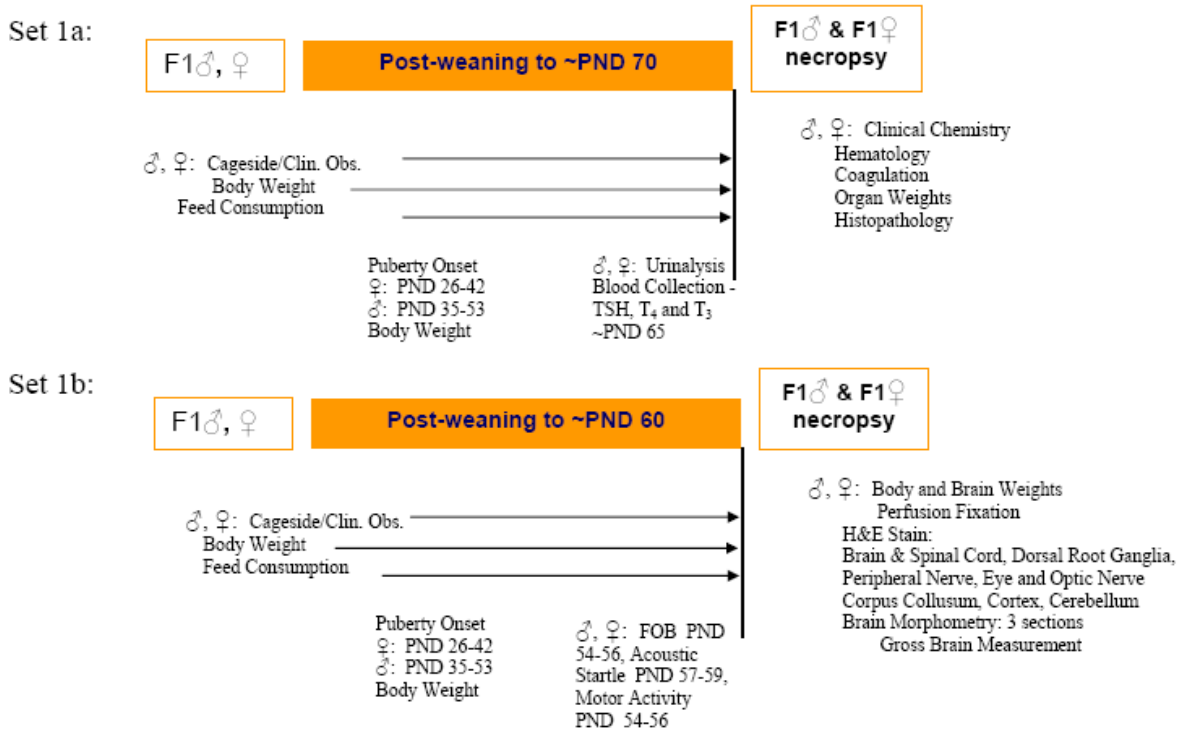


FIGURE 5. Study Design for Set 2 F1 Generation (Immunotoxicity Group)

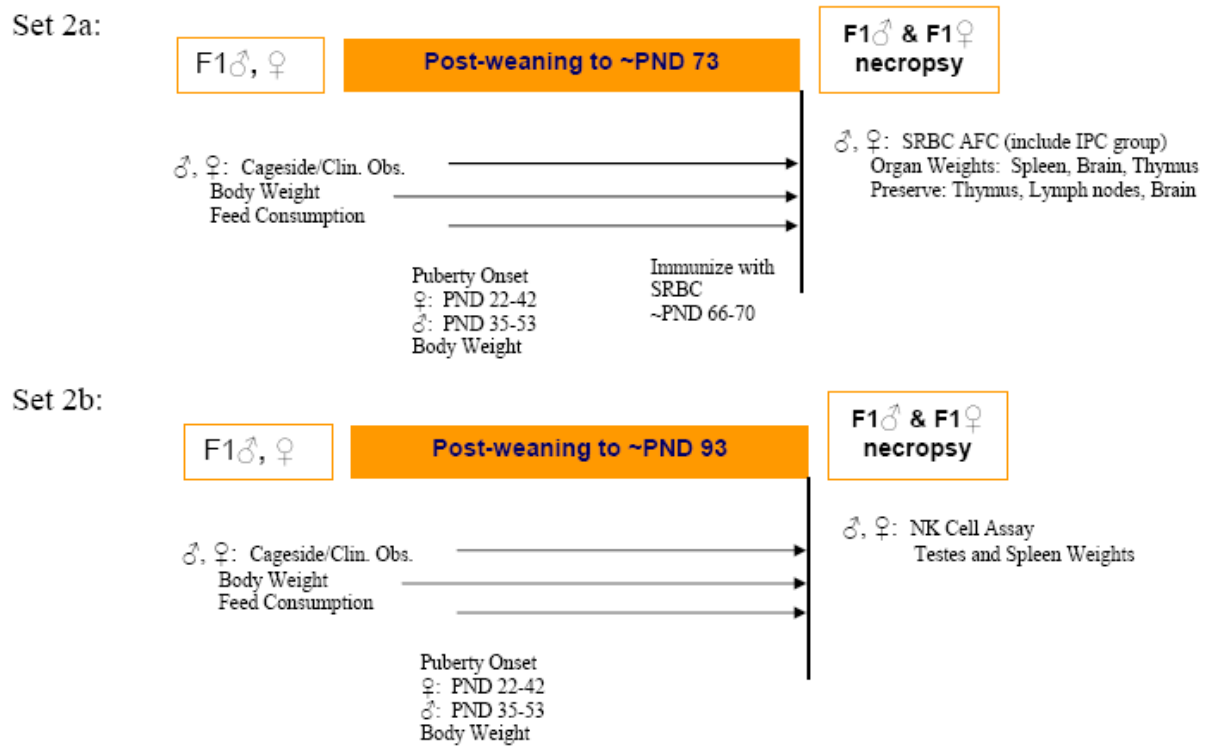
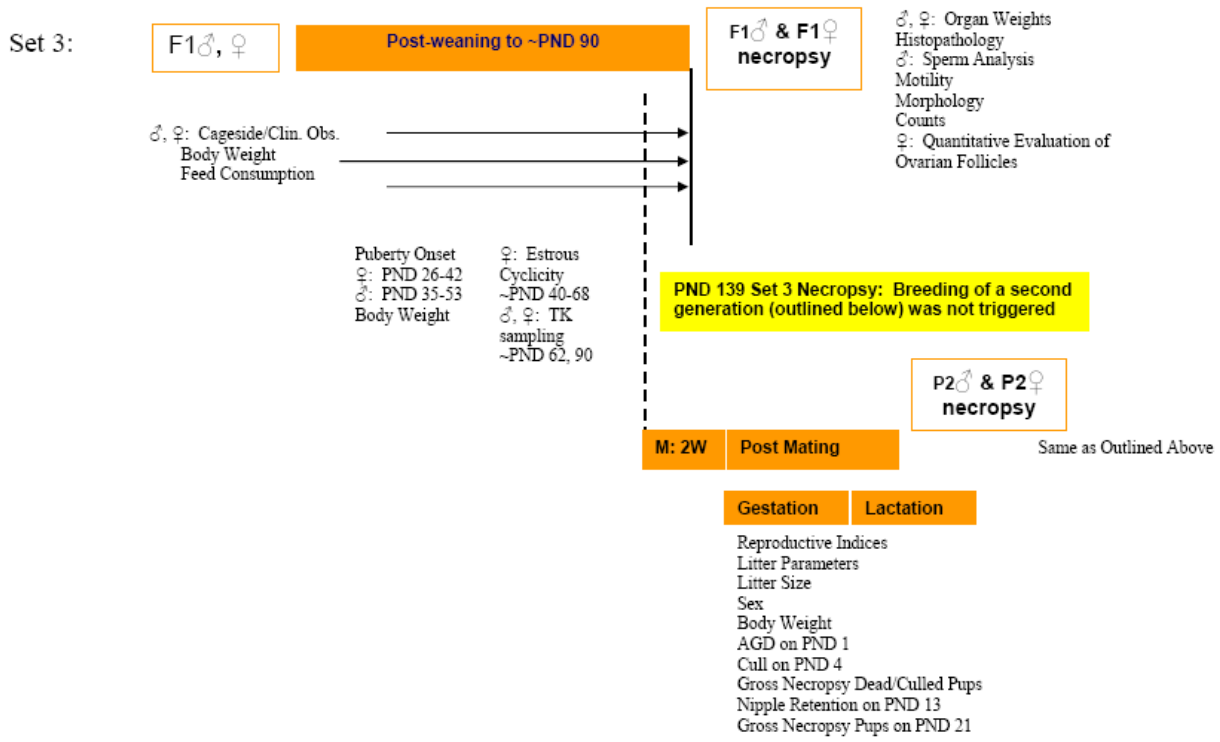


FIGURE 6. Study Design for F1 Generation Set 3 (Reproductive Toxicity Group)



9. **Puberty Onset:** All F1 animals (Sets 1-3) were observed daily for vaginal opening beginning on PND 26 or for balano-preputial separation beginning on PND 35. The presence of vaginal and preputial threads (if any) was noted. Age and body weight of the rats were recorded on the day these markers of puberty onset were acquired. The age and body weight at puberty onset were averaged by litter for data analysis.

10. **Estrous Cycle Evaluation:** The estrous cycle was evaluated in Set 3 F1 females for 4 weeks from ~ PND 40-68 (initiated after vaginal patency). On the day of scheduled necropsy, the stage of the estrous cycle was also determined for the adult F1 female rats (Sets 1a and 3).

ASSESSMENTS COMMON AMONG AGE GROUPS

1. **Hematology, Clinical Chemistry, and Urinalysis:** Blood was collected from 10 rats/sex/dose from P1 males and P1 main study females and Set 1a F1 males and females (both sexes fasted overnight) and GD 17 Satellite dams (not fasted because pregnant). Blood samples were obtained from the orbital sinus following anesthesia with O_2/CO_2 at the scheduled necropsy (after ~11 weeks of exposure in P1 males; on LD 22 in P1 females; on ≈PND 70 in Set 1a F1 offspring). The CHECKED (X) hematological, coagulation, and clinical chemistry parameters were examined.

a. <u>Hematology:</u>

X	Hematocrit (HCT)*	X	Leukocyte differential count*
X	Hemoglobin (HGB)*	X	Mean corpuscular HGB (MCH)*
X	Leukocyte count (WBC)*	X	Mean corpusc. HGB conc.(MCHC)*
X	Erythrocyte count (RBC)*	X	Mean corpusc. Volume (MCV)*
X	Platelet count*	X	Reticulocyte count
	Blood clotting measurements*		
	(Thromboplastin time)		
	(Clotting time)		
X	(Prothrombin time)		

b. <u>Clinical chemistry</u>:

X	ELECTROLYTES	X	OTHER
X	Calcium	X	Albumin
X	Chloride	X	Creatinine
	Magnesium	X	Urea nitrogen
X	Phosphorus	X	Total Cholesterol
X	Potassium	X	Globulins
X	Sodium	X	Glucose
	ENZYMES	X	Total bilirubin
X	Alkaline phosphatase (ALK)	X	Total protein (TP)
	Cholinesterase (ChE)	X	Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase (LDH)		
X	Alanine aminotransferase (ALT/also SGPT)	X	TSH
X	Aspartate aminotransferase (AST/also SGOT	X	T_3
	Sorbitol dehydrogenase	X	T_4
X	Gamma glutamyl transferase (GGT)		
	Glutamate dehydrogenase		

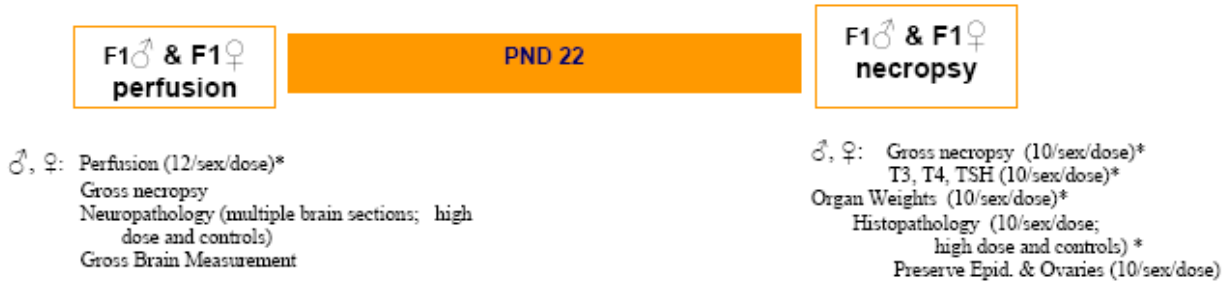
c. Urinalysis: Urine samples were obtained from P1 males and Set 1a F1 offspring (PND 64-66 males and females) during the week prior to the scheduled necropsy. Rats were housed in metabolism cages, and urine was collected overnight (≈16 hours). Feed and water were available during this procedure. The CHECKED (X) parameters were examined.

X	Appearance/color	X	Glucose
X	Volume	X	Ketones
X	Specific gravity/osmolality	X	Bilirubin
X	PH	X	Blood/blood cells
X	Sediment (microscopic)		Nitrate
X	Protein	X	Urobilinogen

2. Toxicokinetics (TK) (GD 17 Satellite dams, Set 3 F1) Blood samples (5/dose group) collected from non-fasted GD 17 dams at necropsy were used for single time point TK analyses to determine blood levels of 2,4-D. These samples were a subset of samples taken for clinical pathology and thyroid hormone assessment. For Set 3 adults, three blood samples (~200 µl) were collected from randomly selected non-fasted animals (5 rats/sex/dose) on PND 63 and PND 84. Blood was collected from the jugular vein without the use of any anesthesia. The first blood sample was collected at 6:00 AM (at the time when lights were turned on), the second blood sample was collected 3 hours later (9:00 AM) and the final blood sample was collected ~1 hour prior to the start of the dark phase of the photocycle (5:00 PM). As demonstrated by Saghir *et al*., (2006), these samples were used to estimate C_{max}, C_{min}, and AUC. Blood was collected from different Set 3 rats at the PND 63 and 84 sampling time points. Immediately after collection, blood samples were centrifuged to separate plasma from red blood cells. Plasma was carefully removed, placed in glass vials and stored in the –80°C freezer until analysis. Stability of 2, 4-D in plasma was established in the previous range-finding study (MRID 47417902).

3. Hormone Measurements, TK Samples, and Clinical Pathology: *Serum for Thyroid Hormone Analyses (Satellite dams, Culled pups, PND 22 Nonperfused Weanlings, Set 1a F1).* Blood from PND 4 culled pups (first 10 litters/dose, if possible; male and female samples separate), PND 22 assigned for termination and standard histopathology (nonperfused), GD 17 satellite dams, and Set 1a F1 offspring (10/sex/dose) was collected under isoflurane anesthesia and placed on ice immediately after collection. Blood from PND 4 same sex pups culled from the same litter were pooled in plastic serum separator tubes and placed on ice. A terminal blood sample from non-fasted GD 17 satellite dams, non-fasted PND 22 weanlings assigned for termination and standard histopathology (non-perfused), and culled pups were used, whereas Set 1a F1 offspring were anesthetized a few days prior to necropsy (~PND 65, non-fasted) for in-life sample collection to avoid the potential effects of fasting on thyroid hormone levels. Serum was separated from cells as soon as possible following blood collection. All sera samples for hormone assays were stored at –80°C. Samples were shipped on dry ice to AniLytics Incorporated (Gaithersburg, Maryland) for analyses of triiodothyronine (T3), thyroxine (T4), and thyroid-stimulating hormone (TSH) using radioimmunoassays. If the volume of serum samples was limited, the priority for hormone analyses was T4, TSH, then T3. Blood samples were not collected from satellite dams that were not noticeably pregnant on GD 17, nor from any PND 22 weanlings not assigned for termination and standard histopathology (nonperfused).

FIGURE 3. Unselected F1 Weanlings



*Using only 1 pup/sex/litter

4. Postmortem Observations (Anatomic Pathology)

a. P1 Adult and Set 3 F1 Offspring: P1 males (fasted) were necropsied after ~ 11 weeks of exposure. Adult P1 females (fasted) were terminated on LD 22 after weaning of their litters, or at least 24 days after the end of the mating period for females with no evidence of mating. F1 male and female adults (fasted) from Set 3 were necropsied on ~ PND 139. Rats were weighed and vaginal lavage smears were prepared from surviving P1 and Set 3 F1 females prior to necropsy. The rats were anesthetized, blood was collected from the orbital sinus (P1 males and females only), and the rats were euthanized by decapitation. A complete necropsy was conducted on all rats. The uteri of all P1 females were stained with an aqueous solution of 10% sodium sulfide stain after removal of the ovaries and examined for the presence and number of implantation sites (*data not provided*). After evaluation, uteri were preserved. Weights of the left testis and left cauda epididymis were collected in P1 males and Set 3 F1 males for use in calculating sperm count parameters. Histopathological examinations were conducted on 10 control and 10 high-dose animals/sex, as well as males and females that failed to mate and/or deliver offspring. The following tissues (X) were prepared for microscopic examination and weighed (XX):

	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT.		NEUROLOGIC
-	Tongue	X	Aorta	XX	Brain (multiple sections)*
X	Salivary glands	X	Heart	X	Peripheral nerve (tibial)
X	Esophagus	X	Bone marrow	X	Spinal cord (3 levels)
X	Stomach	X	Lymph nodes	XX	Pituitary*
X	Duodenum	XX	Spleen*	X	Eyes
X	Jejunum	XX	Thymus♪	X	Cranial nerve (optic nerve)
X	Ileum				GLANDULAR
X	Cecum			XX	Adrenal gland*
X	Colon		UROGENITAL	X	Lacrimal gland
X	Rectum	XX	Kidneys*	XX	Thyroid* b
XX	Liver*	X	Urinary bladder	XX	Parathyroids* b

	Gall bladder	XX	Testes*		
X	Pancreas	XX	Epididymides*		**OTHER**
		XX	Prostate*	X	Bone (including joint)
		XX	Seminal vesicle*a	X	Skeletal muscle
	RESPIRATORY	XX	Ovaries*+	X	Skin
X	Trachea	XX	Uterus* c	X	All gross lesions and masses*
X	Lungs	X	Mammary gland*	X	Oral tissues
X	Nose	X	Vagina*	X	Mesenteric tissues
-	Pharynx	X	Oviducts*	X	Mediastinal tissues
X	Larynx	X	Cervix*		

Data from Table 4, page 230 of the report
* tissues for histopathological examination in P1 rats (both sexes) & Set 3 F1 offspring; ♪ examined in Set 3 F1 offspring
a with coagulating glands and fluids
b Thyroid and parathyroids were weighed together
c with oviducts and cervix

b. Satellite Females: Satellite females (not fasted) were necropsied on GD 17. Rats were weighed, anesthetized, and blood samples were collected by orbital sinus. The tracheas were exposed and clamped, and the animals were euthanized by decapitation using caution not to damage the thyroid glands. A complete necropsy was conducted on all rats. The necropsy included an examination of the external tissues and all orifices. The head was removed, the cranial cavity opened and the brain, pituitary and adjacent cervical tissues were examined. The eyes were examined in situ, and the thoracic and abdominal cavities were opened and the viscera examined. All visceral tissues were dissected from the carcass, re-examined and selected tissues were incised. Weights of the thyroid/parathyroid glands (post-fixation) were recorded, and the thyroid/parathyroid glands were preserved. No other tissues were weighed or saved. Histopathological evaluation of thyroids from control and all dose groups of the GD 17 dams was performed. Gross observations and the number of corpora lutea, implantation sites, and resorptions were recorded. Fetuses were humanely euthanized, examined grossly, and discarded.

c. Culls and Pups Dying or Sacrificed Moribund Before Weaning: Some pups selected for culling on PND 4 were used for thyroid hormone assessment (T3, T4, and TSH). These pups were anesthetized, and blood was collected (heart nick) for serum thyroid hormone analyses. Blood samples were pooled as needed for same sex pups from the same litter. After blood collection, thyroids/parathyroids were collected and preserved for possible future histopathological evaluation. Pups were euthanized by decapitation. Cull pups not selected for thyroid evaluation were euthanized, examined grossly for external and visceral defects, then discarded. Any pup found dead or sacrificed in moribund condition was sexed and examined grossly, to the extent possible, for external and visceral defects, and preserved.

d. PND 22 Weanlings: On PND 22, weanlings (non-fasted) not assigned to Sets 1, 2, or 3 were assigned to a non-perfusion group (1 pup/sex/litter from 10 litters/dose) or a perfusion group (1pup/sex/litter from 12 litters/dose). Gross pathological examination was performed on both non-perfused and perfused selected weanlings. The gross necropsy was performed as described for adults, except that the weanlings were not fasted overnight. Any remaining weanlings not assigned to any of the sets or groups were anesthetized, euthanized by cervical dislocation or decapitation and discarded.

Non-Perfusion Gross Necropsy/Histopathology: PND 22 pups randomly selected at the time of weaning were subjected to a complete necropsy. Pups were anesthetized, weighed, blood collected by orbital sinus or cardiac puncture, and euthanized by decapitation. Organ weights (Table 7) were recorded from 10/sex/dose (representing 10 litters/dose, if possible). The listed organs, and the epididymides and ovaries, as well as relevant gross lesions, were preserved. Slides of kidney, liver, spleen, thyroid, and testis were processed by standard histologic procedures, and histopathological examination of these organs was performed in high-dose and control rats. Relevant gross lesions at all dose levels also were examined. Based on a lack of treatment-related findings at the high dose, no tissues were examined from the lower-dose groups**.**

Table 7. Organs Weights of PND 22 Non-Perfused Weanlings	
Adrenals	Liver
Brain	Spleen
Pituitary (post-fixation)	Thyroid/parathyroid (post-fixation)
Kidney	Testes

Perfusion Neurotoxicity Group (weanlings): Necropsy: Selected weanlings (non-fasted) were injected (*i.p.*) with heparin ≈10 minutes prior to perfusion and anesthetized. While under deep anesthesia, the heart was exposed, the left ventricle cannulated, and the right atrium was incised. Rats were perfused by gravity pressure with phosphate buffer containing sodium nitrite followed by a phosphate-buffered solution of glutaraldehyde – formaldehyde and tissues were examined for gross pathologic findings. The brain (excluding the olfactory lobes), head, spinal column with spinal cord, fore- and hind limbs, and tail were trimmed to remove excessive skin and muscle as needed; muscles from the hind limbs were reflected to further expose the nerves. Tissues listed in Table 7 were saved. **Neuropathology**: Tissues (Table 9 below under DNT Set 1f offspring) were prepared from all rats in all dose groups. Nine cross-sections of the brain were prepared from the following structures: olfactory bulb, cerebrum (frontal, parietal, temporal and occipital lobes), thalamus/hypothalamus, midbrain, pons, medulla oblongata, and cerebellum. In addition, sections were prepared from the trigeminal ganglion and nerve, pituitary gland, eyes with optic nerves, spinal cord (cervical and lumbar), olfactory epithelium, and skeletal muscles (gastrocnemius and anterior tibial). Additionally, sections containing representative sections of the cerebellum and corpus callosum were stained (Luxol Fast Blue) to assess myelin. Spinal nerve roots (cervical and lumbar), dorsal root ganglia (cervical and lumbar), and peripheral nerves (sciatic, tibial (proximal and distal (muscular); at the knee and calf muscle branches) and sural) were osmicated, embedded in epoxy resin, sectioned ≈ 2 to 3 µm thick and stained with toluidine blue. Tissues were evaluated using a light microscope. Histopathologic findings were subjectively graded as appropriate to assess the potential effects of exposure with regard to the contribution of a specific lesion to the health status of an animal. Histopathological examination was performed in high-dose and control rats only, since no relevant gross lesions or treatment-related histopathologic effects were found at the high dose. **Brain Weight and Gross Measurement:** Brain weight and gross linear measurements were recorded on all dose groups. Brains (excluding the olfactory lobes) were weighed following 7-15 days of fixation. Linear measurements consisted of the: 1) cerebral length (L2 – anterior to posterior, excluding olfactory lobes) and width (L3 – maximum), and 2) cerebellar length (L10 – anterior to posterior) and width (L5 – maximum), which were obtained using a hand-held, electronic digital slide caliper (daily calibration).

5. Sperm Analyses: Sperm parameters were evaluated in Set 3 F1 males at termination (same procedures as for P1 males).

DEVELOPMENTAL NEUROTOXICITY (F1 offspring Set 1b)

1. **Functional observational battery (FOB)**: The FOB was conducted between PND 54 and PND 56 under red light conditions by the same observer ("blinded" to the exposure status of the rat) on randomly selected Set 1b F1 rats (10 males and 10 females representing 20 litters) at approximately the same time each test day. The FOB included cage-side, hand-held, and open-field observations, and measurements of body weight, rectal temperature, grip

performance, and landing foot splay (Table 8).

Table 8. FOB Parameters	
Cage-side Observations Abnormal movements or behavior Resistance to removal from cage	**Open-field Observations** Level of activity (ambulatory and rearing) Responsiveness to sharp noise Responsiveness to touch Responsiveness to tail pinch Gait evaluation Urination Defecation
Measurements Rectal temperature Hind limb grip performance Forelimb grip performance grams Landing foot splay	
Hand-held Observations Palpebral closure Lacrimation (non-colored periocular wetness) Pupil size Pupil reactivity Salivation (non-colored perioral wetness) Muscle tone Extensor-thrust response Reactivity to handling	**Categorical Observations** Abnormal behavior Abnormalities of the eye Abnormal urine or feces Abnormalities of the gastrointestinal (GI) tract Injury Missing extremity Abnormal muscle movements Palpable mass/swellings Abnormal posture Abnormalities of the reproductive system Abnormal respiration Abnormal skin or hair-coat/mucous membranes Excessive soiling General abnormalities

a. **Hand-Held and Open-Field Observations:** Hand-held and open-field observations included a careful physical examination and sensory evaluation according to an established format. Open-field observations and sensory evaluations were made in a clear plastic box (50 cm × 50 cm). The rat was placed in the center of a 20-cm diameter circle marked on the bottom of the open-field box. Activity was defined as ambulatory activity including rearing. Non-ambulatory movements, such as grooming and exploratory sniffing, were not a part of this evaluation. If present, abnormal movements (i.e., tremors, convulsions), or behaviors (i.e., stereotypes), were recorded.

b. **Rectal Temperature:** Rectal temperature was measured by carefully placing a rectal thermistor ≈ 4 cm into the rectum for ≈10 seconds to record temperature. The thermistor was validated at 37°C before, during and after the study.

c. **Grip Performance:** Hind limb grip performance was tested according to the procedure described by Mattsson et al. (1986). Briefly, the observer placed the rat's forepaws on a plastic bench and the hind paws were set on a horizontal screen attached to an electronic strain gauge. The observer then smoothly but firmly pulled backward on the tail until the rat's grip on the screen was broken. An electronic strain gauge was used to record the rat's resistance to the pull in grams. The average of three trials was used for statistical analysis. Forelimb grip performance was similarly tested. In this application, a bench was not used, and the rats were placed so that the forepaws were on the screen and the hind paws were suspended ≈10 cm above the plastic platform.

d. **Landing Foot Splay:** The outermost toe of each hind paw was marked with ink, and the rat was dropped from a height of 30 cm onto the recording sheet. This procedure was repeated three times (toes re-inked, as necessary). The distance from center-to-center of the ink marks, for each trial, were measured (cm) and the average of the three landing splay values was used for statistical analysis.

e. **Motor Activity:** An automated system was used for motor activity (MA) data collection on 10 Set 1b rats/sex/dose between PND 54 and 56. Each test session consisted of eight 8-minute epochs, totaling 64 minutes of testing per rat per test session. This duration was chosen based on the results of a validation study indicating that performance of control rats approached asymptote in 50-60 minutes in Crl:CD(SD) rats (Marty and Andrus, 2007). Activity counts for each epoch were recorded. Data were presented as total motor activity counts and counts/epoch. Rats were allocated to the motor activity cages in such a way that the counterbalancing of exposure groups and sexes across cages and test times was maximized.

f. **Acoustic Startle Response (ASR) Habituation System Description**: The ASR of the F1 Set 1b rats was tested on PND 57-59. Body weights were collected on the day of ASR assessment. The ASR system was a commercially available package consisting of 8 acoustically insulated chambers and hardware to generate the auditory stimuli and measure the resulting startle responses. Each chamber contained a dual-speaker enclosure (for the generation of background noise and audio stimuli), a load-sensing platform, and an adjustable gain preamplifier. The startle stimulus speaker had a frequency response of 1.8 kHz - 30 kHz, and the background noise speaker was had a frequency response of 40Hz - 8kHz. Each acrylic animal holder was attached to a load-sensing platform that was connected to the data acquisition system. Each chamber was equipped with a red house light, which was kept on during all experimental procedures.

On the day of testing, each rat was allowed to acclimate (≈ 30 minutes) in the testing area in a transfer cage before being placed into an acrylic animal holder, which was then attached to a load cell platform inside the chamber. Following a 15-minute acclimation period in the presence of background noise (~65 dB[A]), rats received 50 startle trials. Each trial consisted of a burst of white noise (~120 dB[A], 50-msec duration, 2-msec rise/fall time), and trials were separated by a variable inter-trial interval (10-20 seconds). The first positive peak occurring within 250 msec following the startle stimulus (minimum latency 20 msec, minimum peak amplitude 20) was defined as the peak startle response. The trials were analyzed in blocks of 10 to examine habituation**.**

2. Necropsy/Neuropathology: On PND 60, F1 offspring Set 1b male and female rats selected for perfusion were counterbalanced across dose groups and given an *ip* injection of heparin ≈10 minutes prior to perfusion. Rats were perfused by gravity pressure with phosphate buffer containing sodium nitrite followed by a phosphate-buffered solution of 1.5% glutaraldehyde–4% formaldehyde. Tissues were examined for pathologic alterations. The brain (excluding the olfactory lobes), head, spinal column with spinal cord, fore-and hind limbs, and tail were trimmed to remove excessive skin and muscle; muscles from the hind limbs were reflected to further expose the nerves.

Tissues for neuropathologic evaluation (Table 9) were prepared from all rats in the

control and high-dose groups. In addition, appropriate brain sections from the low- and intermediate-dose groups were taken to slide for possible morphometric evaluation. Nine cross-sections of the brain were prepared using a hand-held, single-edged industrial blade from the following structures: olfactory bulb, cerebrum (frontal, parietal, temporal and occipital lobes), thalamus/hypothalamus, midbrain, pons, medulla oblongata, and cerebellum. For morphometric purposes, two transverse tissue blocks were cut through the cerebrum and midbrain (block #3 and #4). A longitudinal (anterior to posterior) cut was made midway through the cerebellum after it was removed from the midbrain (block #10). The following gross rostral landmarks for each block were used: block #3 –optic chiasm, block #4 – anterior edge of infundibulum, and block #10 dorsal midline apex of the cerebellum. Blocks #3 and #4 would contain the following structures: cerebrum (frontal and parietal lobes), thalamus/hypothalamus, and midbrain. These tissues were processed by standard histologic procedures, embedded in paraffin, sectioned approximately 6-µm thick and stained with hematoxylin and eosin, and coverslipped.

Table 9. Nervous System Tissue Collection

Brain	**Dorsal Root Ganglia**
►Olfactory bulb	►Cervical and Lumbar
►Cerebrum, frontal, parietal, temporal, occipital	**Dorsal and Ventral Roots**
►Thalamus/Hypothalamus	►Cervical and Lumbar
►Midbrain	**Peripheral nerves** (cross and longitudinal section)*
►Pons	►Proximal sciatic
►Cerebellum	►Proximal & distal tibial (at knee & calf muscle branches)
►Medulla including nucleus gracilis/cuneatus	►Peroneal (saved)
Pituitary gland	►Sural
Trigeminal ganglia with nerve	►Caudal (saved)
Spinal cord (cross and oblique section)	Nasal tissue with olfactory epithelium
►Cervical Swelling (C3-C6)	Eyes - with optic nerve (longitudinal section only)
►Lumbar Swelling (L1-L4)	**Skeletal muscle**
	►Anterior tibial and Gastrocnemius

Data from Table 5, page 233 of the report. *Fore- and hind limbs saved, but nerves collected only from the right hind limb.

A sufficient number of sections were cut from each block to meet the criteria of finding the appropriate microscopic landmarks for the purpose of measuring specific microscopic structures. Microscopic landmarks were as follows: block #3 (Figure 8) – first occurrence of the anterior commissure, block #4 (Figure 9) – contact/close proximity of the dentate gyrus and the polymorph layer of the dentate gyrus, and block #10 (Figure 10) – midline longitudinal section that contains the medial cerebellar nucleus. The most appropriate section from each block was used for the microscopic measurements. Additional sections from blocks #3, #4, and #10 from the brain were stained with Luxol Fast Blue and cover-slipped to assess myelin. In addition, sections were prepared from the trigeminal ganglion and nerve, pituitary gland, eyes with optic nerves, spinal cord (cervical and lumbar), olfactory epithelium, and skeletal muscles (gastrocnemius and anterior tibial). These tissues were processed by standard histologic procedures, embedded in paraffin, sectioned approximately 6-µm thick and stained with hematoxylin and eosin, and coverslipped. Spinal nerve roots (cervical and lumbar), dorsal root ganglia (cervical and lumbar), and peripheral nerves (sciatic, tibial (proximal and distal (muscular) – at the knee and calf muscle branches) and sural) were osmicated, embedded in epoxy resin, sectioned ≈ 2 to 3 µm thick and stained with toluidine blue.

Tissues were evaluated using a light microscope. Histopathologic findings were subjectively graded as appropriate to assess the potential effects of exposure with regard to the contribution of a specific lesion to the health status of an animal. The report indicated that treatment-related effects were not observed in any neurological tissues in high-dose rats; therefore, slides from peripheral nervous system tissues were not prepared from the low- and intermediate-dose level rats. Aside from sections #3, #4, and #10, slides from other brain sections (Figure 7 in report) were not prepared due to the lack of treatment-related effects in the high-dose group. Sections #3, #4, and #10 (brain morphometry sections) were prepared for low- and intermediate-dose level rats to avoid potential temporal artifacts from retention in paraffin blocks.

a. Brain Weight and Gross Measurements - Brain weight and gross linear measurements were recorded on all dose groups. Brains (excluding the olfactory lobes) were weighed following 7-15 days of fixation. Linear measurements consisted of the: 1) cerebral length (L2 – anterior to posterior, excluding olfactory lobes) and width (L3 – maximum), and 2) cerebellar length (L10 – anterior to posterior) and width (L5 – maximum), which were obtained using a hand-held, electronic digital slide caliper, whose calibration was checked prior to use each day (Figures 11 and 12).

b. Morphometrics - Microscopic Brain Measurements. Microscopic brain measurements were recorded on all rats in the control and high-dose groups. Microscopic brain measurements were obtained from a number of anatomical structures in tissue sections stained with H&E from blocks #3, 4, and 10 having the appropriate landmarks. Images of each section of brain were digitally captured using an Olympus DP70 or a SPOT RT camera mounted on a Leitz dissecting microscope (blocks # 3 and 4; Figures 8 and 9) or a Leitz light microscope (block #10; Figure 10) with image capture software (Diagnostic Instruments, Inc., Sterling Heights, Michigan). Simple morphometric measurements were obtained using the Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, Maryland) as follows: 1) distance measurement - creates a measurement of the distance between two user-selected features, and 2) curve measurement - creates a measurement of the average distance between two user-selected traces in any direction. Measurements were excluded as necessary due to microscopic artifacts, such as missing portion(s) of tissue, tears in tissue etc. Morphometric data obtained using the Image-Pro Plus was electronically transferred to Microsoft Excel spreadsheets.

DEVELOPMENTAL IMMUNOTOXICITY (F1 offspring Set 2)

1. **Sheep Red Blood Cell (SRBC) Antibody-Forming Cell (AFC) Assay Immunotoxicity evaluations**: To obtain data on the functional responsiveness of the immune system, Set 2a F1 rats (10/sex/dose from 10 litters) were immunized four days prior to necropsy (PND 66 to 70) with a single, 0.5 ml intravenous (i.v.) injection of 2 x 10^8 SRBC, via the lateral tail vein. In addition, five rats/sex from similarly aged satellite rats (extra rats in laboratory) served as a concurrent positive control (IPC) group, which was also immunized four days prior to necropsy. These latter rats were administered a daily dose of 20 mg/kg/day cyclophosphamide (CP; causes reduction in the anti-SRBC antibody forming cells (AFC) response) *via* intraperitoneal (i.p.) injection for the four days prior to necropsy. On the day of necropsy (~PND 67-73), all rats were euthanized *via* CO_2 anesthesia followed by cervical dislocation and removal of the spleens. The

spleens were trimmed, weighed and placed into homogenization bags containing RPMI. A single cell suspension of the spleen cells was prepared by gentle disaggregation using a tissue homogenizer. The homogenized spleen suspensions were filtered through a 100 µm cell strainer (VWR catalog #21008-952) into labeled 50 ml centrifuge tubes. Each bag was rinsed with ≈2 ml of 5% RPMI and the rinse transferred to the appropriate centrifuge tube. The tubes containing the spleen cell suspensions were centrifuged at 300 x g for 10 minutes at 4°C. The supernatant was discarded. The cells were washed once by re-suspending the pellet in 10 ml of 5% FBS/RPMI and centrifuged (as above) and the supernatant again discarded. The cells were then re-suspended in 5 ml of 5% FBS/RPMI. A 1:1000 dilution of the spleen cells was prepared in 10 ml of Isoton II with three drops of Zap-Oglobin II lytic reagent and the number of cells was counted using a Coulter Z1 cell counter (Beckman Coulter, Miami, Florida). Three additional dilutions of spleen cells were prepared (e.g., 1:50, 1:100, or 1:200) in 5% FBS/RPMI. The diluted cells were mixed with washed SRBC (same lot used for immunization), guinea pig complement, and warm agar with DEAE-dextran. The mixture was poured into the middle of a Petri dish. A humidified (breath) coverslip was placed on the mixture and after one or two minutes, the dish was placed into a humidified incubator set at 37°C for 3 hours. The dishes were removed from the incubator and the plaques were counted. *Plaques are formed as a result of complement-mediated lysis of the SRBC from antibodies secreted by the plasma cells (differentiated B cells).*

2. **Natural Killer Cell (NK) Assay:** Immunotoxicity assessment of the remaining rats in Set 2 (Set 2b: 10/sex/dose from 10 litters) was determined after evaluation of the preliminary AFC data. Set 2b rats were evaluated using the natural killer cell (NK) assay on PND 87-93 for effects on the immune system that are more cellular in nature. Mononuclear lymphoid cells from the spleen were obtained from Set 2b rats maintained on 2, 4-D diet continuously through PND 87-93. The spleens were surgically removed and a single cell suspension was prepared by gentle disaggregation using a tissue homogenizer. Mouse lymphoma cell line YAC-1 (ATCC) was used as target cells. YAC-1 cells were subcultivated prior (~48 hours) to NK incubation to develop a growth phase population of target cells. YAC-1 cells were labeled with carboxy-fluorescein succinimidyl ester (CFSE) just prior to mixing with spleen cells (effectors) at effector:target ratios that range from 6.25:1 to 200:1. Note: The effector to target ratios in this study ranged from 50:1 to 800:1. The samples were incubated for 4 hours at 37°C with 5% CO_2. At the end of incubation, propidium iodide (PI) was added and incubated at 4°C for 5 minutes to identify dead cells. Rabbit anti-mouse/rat Asialo GM1 polyclonal antibody was used as a positive control. A separate group of age-matched rats received a single intravenous injection of the positive control 24 hours prior to necropsy. The samples were analyzed by flow cytometry within one hour after adding PI. Comparative assessments of the flow cytometry and chromium release approach indicate a similar sensitivity in their ability to detect NK cell activity and inhibition. The present evaluation revealed linear increases in cytotoxicity with increasing effector to target cell ratios (from 5 to 40% cytotoxicity) and significant inhibition with the positive control anti asailo-GM1 (greater than 50% inhibition); responses that were similar to that reported for the chromium release approach. The study authors stated that advantages of the flow cytometry approach to the NK-cell assay include higher signal, lower spontaneous release of signal, and avoidance of isotope waste and expense. The use of flow cytometry for the NK-cell evaluation as a non-guideline study was approved by the USEPA prior to

the initiation of this procedure. It is to be noted that the flow cytometry for NK cell activity assay has not been approved as an alternative assay vs ^{51}Cr-release NK assay for the Guideline Immunotoxicity Study (OPPTS 870.7800) until further validation has been completed.

3. **Necropsy/Anatomic Pathology (Set 2 F1 Offspring):** A limited necropsy was conducted on Set 2a rats (10 rats/sex/dose). Terminal body weights, spleen, brain and thymus weights were collected and organ:body weight ratios were calculated. Spleens were used for the SRBC AFC assay. The thymus and mesenteric and mediastinal lymph nodes were collected and preserved for possible future histopathological examination. In addition, the brain also was preserved. The remaining F1 Set 2 rats (Set 2b; 10/sex/dose) used for an NK assay on PND 87-93 were weighed and anesthetized (CO_2 inhalation) followed by decapitation. Only terminal body weights, spleen, and testes weights were collected. Spleens were used for the NK assay. The testes were preserved for possible future histopathological examination.

D. DATA ANALYSIS

1. **Statistical Analyses:** Appendix Table A summarizes the statistical analyses performed. Averages and standard deviations were calculated for the brain weights and morphometric measurements using Microsoft Excel spreadsheets and databases in full precision mode (15 digits of accuracy). Means and standard deviations were calculated for all continuous data. Descriptive statistical (*i.e.,* mean ± standard deviation) analyses of toxicokinetic data were performed using Microsoft Excel (Microsoft Corporation, Redmond, Washington) spreadsheets in the full precision mode (15 digits of accuracy). Individual values obtained were analyzed for $AUC_{0\rightarrow t}$ by trapezoidal method using PK Functions for Microsoft Excel. (J. I. Usansky, A. Desai, D. Tang-Liu, Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, California; Pizarro *et al*., 2004; Beringer *et al*., 2005). Whenever statistical analysis was needed to compare trends among doses, data was analyzed using SAS statistical analysis computer software (SAS Institute Inc., Cary, North Carolina) through a two-way analysis of variance. Linearity was determined graphically via regression analysis (as per Sweatman and Renwick, 1980; Roberts and Renwick, 1989) as well as by comparing ratios of plasma 2, 4-D and AUC_{24h}. If deemed necessary, additional statistical tests were performed. Because numerous measurements were statistically compared in the same group of animals, the overall false positive rate (Type I errors) was greater than the nominal alpha levels. Therefore, the final toxicologic interpretation of the data considered other factors, such as dose-response relationships, biological plausibility and consistency, and historical control values.

2. **Indices:** Reproductive indices (all dose levels in P1 rats) and offspring viability indices were calculated as follows (as provided in the study report):

   a. Reproductive indices:

   - Female mating index = (No. females with evidence of mating/No. paired) x 100
   - Male mating index = (No. males with evidence of mating/No. paired) x 100
   - Female conception index = (No. females with evidence of pregnancy/No. mated) x 100
   - Male conception index = (No. males siring a litter/No. mated) x 100

- Female fertility index = (No. females with evidence of pregnancy/No. paired) x 100
- Male fertility index = (No. males siring a litter/No. paired) x 100
- Gestation index = (No. females delivering viable litter/No. females with evidence of pregnancy) x 100
- Gestation survival index = percentage of delivered pups alive at birth

b. Offspring viability indices:

- Post-implantation loss = (No. implants – No. viable offspring)/(No. implants) x 100
- Day 1 or 4 pup survival index = (No. viable pups on day 1 or 4/No. born live) x 100
- Day 7, 14, or 21 pup survival index = (No. viable pups on day 7, 14 or 21/No. after culling) x 100

3. **Historical control data:** Historical control data were provided for Acoustic Startle Response (ASR) from recent control data sets from performing laboratory in male rats of the same strain, age, and body weight; for male reproductive and sex accessory gland weights; for male pituitary weights; testis size, bladder calculi. A dose range-finding study (MRID 47417901) was submitted that provides additional data, and a separate abbreviated DER is appended.

II. RESULTS

A. PARENTAL ANIMALS

1. **Mortality and clinical signs:** There were no treatment-related deaths or clinical signs of toxicity in either sex. All P1 rats survived to scheduled necropsy.

2. **Body weight and food consumption:** Body weight was not adversely affected in the P1 male and female rats during the pre-mating period (Table 10), and body-weight gains were comparable among the groups (both sexes). During gestation, there were no treatment-related effects on body weight or body-weight gain (Table 11) in the main study females. During lactation, female body weights were slightly but significantly decreased (↓5%) on lactation day 7 (LD7) in the high-dose group just prior to the diet adjustment of 2,4-D concentrations (1/2 concentration female diets given on LD 7-14 to account for the large increase in maternal feed consumption during this period). By LD 14, body weights were similar to the control group. Body-weight gain was reduced at the 600 ppm dose level during LD 1-4 (↓64%*) and LD 4-7 (↓50%) but was greater than control during LD 7-14 (Table 11). NOTE: The standard deviation in body-weight gain values during lactation are greater than the mean in several instances (all groups), and the 300 ppm dose group displayed a greater reduction in body-weight gain during LD 4-7 (↓79%) than the 600 ppm dose group at either time point. Food consumption was comparable among the groups during pre-mating (both sexes) and in females during gestation. During the first week of lactation, the high-dose dams consumed less food (LD 1-4: ↓10%; LD 4-7: ↓7%) than the control dams.

TABLE 10. Mean (±SD) Body weight, body-weight gain, and food consumption - pre-mating [a]

Observations/study week	Dose group			
	Control	100 ppm	300 ppm	800/600 ppm
P1 Males - Pre-mating				

TABLE 10. Mean (±SD) Body weight, body-weight gain, and food consumption - pre-mating [a]

Observations/study week	Dose group			
	Control	100 ppm	300 ppm	800/600 ppm
Mean body weight (g)				
Day 1	339.7±12.4	344.2±10.6	344.9±11.9	343.1±11.0
Day 78	548.2±43.9	548.2±49.2	550.3±51.3	560.7±45.2
Mean weight gain (g)				
Days 1-8	38.5±9.3	36.0±8.0	38.8±9.8	40.5±9.9
Days 1-78	208.5±37.1	204.0±42.4	205.3±42.7	217.6±39.4
P1 Females – Pre-mating				
Mean body weight (g)				
Day 1	231.1±9.5	231.3±8.1	232.9±9.4	232.1±11.0
Day 29	266.3±19.2	269.9±17.1	272.1±21.0	267.8±19.1
Mean weight gain (g)				
Days 1-8	13.6±7.1	16.0±7.6	12.2±9.0 (↓10%)	12.3±7.5 (↓10%)
Days 1-29	35.2±13.3	38.7±12.0	39.2±14.5	35.7±13.0

[a] Data obtained from Table 17 (pages 248-249) and Table 18 (page 250) in the study report.
* p<0.05; ** p<0.01. n = 27 (males)/39 (females; main +satellite)

TABLE 11. Mean (±SD) Body weight and body-weight gain – Gestation and Lactation[a]

Observations/study week	Dose group			
	Control	100 ppm	300 ppm	800 ppm
P1 Females – Gestation				
Mean body weight (g)	n = 26	n = 27	n = 24	n = 24
Day 0	268.4±19.9	273.4±16.8	276.7±23.1	269.4±18.6
Day 7	306.0±20.8	308.7±16.6	310.0±25.8	302.4±19.4
Day 20	414.3±31.4	416.6±30.2	417.5±34.5	416.0±32.9
Mean weight gain (g)				
Days 0-7	37.6±12.6	35.3±7.6	33.3±8.3	32.9±6.3 ↓12%
Days 0-20	145.9±25.8	143.2±24.0	140.8±18.0	146.6±20.4
P1 Females – Lactation				
Mean body weight (g)	n = 27	n = 28	n = 24	n = 24
Day 1	317.4±21.8	318.3±20.8	317.6±23.8	312.7±23.1
Day 7	336.6±14.8	333.3±20.2	330.9±23.8	320.2±21.7*↓5%
Day 21	335.2±19.0	336.8±18.4	341.5±22.2	335.6±25.0
Mean weight gain (g)				
Days 1-4	15.4±10.3	10.9±7.7↓29%	12.5±9.6 ↓19%	5.5±11.6* ↓64%
Days 4-7	3.8±7.5	4.2±7.6	0.8±7.9 ↓79%	1.9±9.8 ↓50%
Days 7-14	12.8±12.5	14.3±13.5	14.0±13.4	26.5±14.7*
Days 1-21	17.8±20.8	18.5±15.4	23.8±12.3	22.9±12.1

[a] Data obtained from Tables 19, 21, 23, 24 (pages 251, 253, 255, 256) in the study report; * p<0.05; ** p<0.01.

3. **Test material intake:** Test material intake (TMI, expressed as mg/kg/day) was calculated using feed concentrations, body weights, and feed consumption data and is shown in Table 12. The TMI values for the P1 males were fairly close to the predicted values (i.e., 5.5 vs. 5 mg/kg/day; 16.6 vs. 15 mg/kg/day and 45.3 vs. 40 mg/kg/day). However, in the P1females, TMI exceeded the targeted low and high dose levels of 5 and 30 mg/kg/day, particularly during the early lactation period (LD 1-7), when TMIs were double these targeted dose levels.

TABLE 12. Mean test substance intake during premating, gestation, and lactation (mg/kg body weight/day) [a]						
	P1 Males			P1 Females		
	100 ppm	300 ppm	800 ppm	100 ppm	300 ppm	600 ppm
Premating	5.51±0.68	16.6±2.08	45.3±5.73	7.0±0.42	20.6±0.65	40.2±1.18
Gestation ●Main study ●Satellite	-	-	-	7.37±0.77 7.63±0.55	21.9±2.14 22.9±1.17	43.7±4.02 45.3±2.65
Lactation (main) ●LD 1-7[B] ●LD 7-14[B]	-	-	-	10.7 8.4	32.4 25.5	64.5 51.5

[a] Data obtained from Text Table 24 (page 136), Table 32 (males), page 264, Tables 34-35 (females), pages 266-267 in study report.
[B] interval standard deviation not provided

4. Hematology – P1 Males and Main Study Females

There were no apparent treatment-related effects on hematology, differential white blood cell counts, and prothrombin time in either sex at any dose level. Table 13 provides the platelet count findings for comparison to those of the F1 offspring Set 1a males who displayed a reduction in platelet counts (↓14%) on PND 70.

Table 13. Platelet Counts – P1 Adults				
Parameter	**0 ppm**	**100 ppm**	**300 ppm**	**600/800 ppm**
Males				
Platelet counts (E3/UL)	1130±146	1060±102	1165±99	1058±150 ↓6%
WBC (E3/UL)	10.87±2.10	10.05±2.39	9.42±1.74 ↓13%	11.39±2.17
Females				
Platelet counts (E3/UL)	1250±271	1225±246	1234±152	1237±159
WBC (E3/UL)	9.98±3.44	10.27±3.49	10.25±1.80	8.32±2.30 ↓17%

Data from Tables 39 and 40, pages 272 and 273; Tables 43 and 44, pages 276 and 277 of report; n=10

5. Clinical chemistry – P1 Males and Main Study Females

At 800 ppm, P1 males displayed statistically significant (slight) decreases in total protein (↓6%) and albumin (↓5%) levels compared with controls and non-significant increases in ALT (↑80%) and AST (↑26%). The increases in ALT and AST were due to one outlier. P1 females did not display any treatment-related changes in any of the clinical chemistry parameters monitored.

6. Urinalysis – P1 Males

There was a slight, dose-related increase in white blood cells per microscopic field, which was noted at 300 and 800 ppm during the urine microscopic examinations. All other parameters were comparable among the groups.

7. Macroscopic examination – P1 Males and Main Study Females

Males: Two males [one at 300 ppm (#4310) and one at 800 ppm (#4334)] had decreased testis size (bilateral) and no motile sperm. The 300 ppm male also had a decreased seminal vesicle size (unilateral). Two males [one at 300 ppm (#4311) and one at 800 ppm (#4338)] had calculi in the urinary bladder. This resulted in diffuse thickening of the bladder wall in these rats. F**emales**: There were no gross observations attributed to 2, 4-D in the P1 females.

8. Organ weights – P1 Males and Main Study Females

Males: There were no effects on terminal body weights in the P1 males following ≈11 weeks of exposure (Table 14). Similarly, there were no significant, treatment-related effects on absolute or relative liver, brain, spleen, thyroid, pituitary or adrenal gland weights. There was a significant increase in absolute (13%) and relative (11%) kidney weights of P1 males at 800 ppm. Kidney weights were not significantly affected at 100 ppm or 300 ppm. The kidney-weight changes were accompanied by histopathological findings in the kidneys (see below). Absolute seminal vesicle weights were significantly decreased at both 300 and 800 ppm (↓12%). Relative seminal vesicle weights also were significantly decreased by 12% and 14%, respectively. There was a dose-related decrease in absolute and relative prostate weights in males at the 800 ppm (10% and 11%, respectively) and 300 ppm (9% and 8%, respectively), but these decreases were not statistically significant. It is noteworthy that the control values for relative organ weights of the seminal vesicles, prostate, testes and epididymides in the P1 males were outside of the laboratory historical control range (Table 15), suggesting that the control values were atypical. The organ weights for the 800 ppm-treated males were within the laboratory historical control range. Absolute and relative prostate and seminal vesicle weights also were within historical control values for the 300 ppm-exposed males. There were no treatment-related effects on testes or epididymal weights. Additionally, there was no associated histopathology in P1 male reproductive or accessory sex gland tissues. These changes in male reproductive organ weight were considered spurious because no consistent pattern of altered androgenicity was observed in male rats treated with 2, 4-D, no significant changes on sperm parameters (spermatid/sperm counts, sperm motility and sperm morphology) were found, and lower accessory sex gland weights were not observed in the F1 offspring (Set 1a or Set 3) with longer 2, 4-D exposures (*in utero*, during lactation and into adulthood). **Females** (LD 22): No treatment-related effect was observed on terminal body weight in P1 females. There were no significant, treatment-related effects on absolute or relative liver, kidney, brain, spleen, ovary, pituitary, or adrenal gland weights. Uterine weight (↑17% both absolute and relative) was increased at 600 ppm, but statistical significance was not attained. However, increased uterine weights were observed in Set 1a and Set 3 F1 offspring also. Relative thyroid weights were statistically significantly decreased (↓11%) at 300 ppm, but absolute thyroid weights (↓11%) were not statistically significant, and no dose response was evident. NOTE: The thymus was not weighed in this group of rats, but decreased thymus weights were observed in the offspring at PND 70 and PND 139.

TABLE 14. Organ Weights[a] P1 Main Study Rats

Organ	Dose group (ppm)			
	Control	100	300	600/800
P1 MALES				
Terminal Body Weight (g) N=	529.7±45.4 27	528.8±50.8 27	530.627±51.0 27	540.7±46.8 27
Kidneys ●absolute ●relative	 3.513±0.532 0.662±0.070	 3.621±0.440 0.686±0.064	 3.629±0.496 0.685±0.083	 3.965±0.488* ↑13% 0.734±0.068* ↑11%
Liver ●absolute ●relative	 14.006±1.794 2.638±0.154	 14.002±2.048 2.641±0.217	26 14.035±1.635 2.661±0.184	 14.403±2.084 2.655±0.206
Thyroid ●absolute ●relative	 0.0240±0.0048 0.0045±0.0009	 0.0235±0.0033 0.0045±0.006	 0.0234±0.0031 0.0044±0.0005	 0.0253±0.0044 0.0047±0.0008
Testes ●absolute ●relative	 3.718±0.229 0.706±0.060	 3.654±0.247 0.696±0.075	 3.595±0.551 0.686±0.127	 3.528±0.501 ↓5% 0.653±0.086 ↓8%
Prostate ●absolute ●relative	 1.313±0.174 0.248±0.029	 1.274±0.232 0.242±0.047	 1.198±0.228 ↓9% 0.228±0.052 ↓8%	 1.188±0.257 ↓10% 0.221±0.053 ↓11%
Seminal vesicles ●absolute ●relative	 1.887±0.221 0.358±0.045	 1.811±0.345 0.347±0.078	 1.653±0.297* ↓12% 0.315±0.067* ↓12%	 1.655±0.297* ↓12% 0.308±0.059* ↓14%
Epididymides ●absolute ●relative	 1.498±0.106 0.285±0.033	 1.428±0.110 0.272±0.029	 1.405±0.165 ↓6% 0.268±0.042 ↓6%	 1.418±0.154 ↓5% 0.263±0.030 ↓8%
Brain ●absolute ●relative	 2.126±0.106 0.404±0.037	 2.129±0.085 0.406±0.040	 2.160±0.091 0.410±0.036	 2.154±0.089 0.401±0.031
Adrenal ●absolute ●relative	 0.055±0.010 0.010±0.002	 0.059±0.010 0.011±0.002	26 0.056±0.008 0.011±0.002	 0.058±0.009 0.011±0.002
Pituitary ●absolute ●relative	 0.0136±0.0015 0.0026±0.0002	 0.0137±0.0015 0.0026±0.0003	 0.0132±0.0020 0.0025±0.0003	 0.0135±0.0014 0.0025±0.0003
P1 FEMALES (LD 22)				
Terminal Body Weight (g) n=	295.8±15.1 27	297.5±16.6 28	300.5±23.6 24	296.5±19.3 24
Kidneys ●absolute ●relative	 2.328±0.192 0.789±0.078	 2.275±0.215 0.766±0.077	 2.417±0.159 0.807±0.062	 2.395±0.272 0.809±0.086
Liver ●absolute ●relative	 12.538±1.241 4.244±0.423	 12.081±1.303 4.061±0.375	 12.784±1.352 4.253±0.264	 12.130±1.179 4.089±0.258
Thyroid ●absolute ●relative	 0.0180±0.0029 0.0061±0.0010	 0.0168±0.0021 0.0057±0.0007	 0.0162±0.0028 0.0054±0.0009*↓12%	 0.0172±0.0021 0.0058±0.0008
Ovaries ●absolute ●relative	 0.113±0.024 0.038±0.008	 0.118±0.027 0.040±0.009	 0.119±0.029 0.039±0.009	 0.111±0.023 0.037±0.008
Uterus ●absolute ●relative	 0.677±0.169 0.229±0.056	 0.668±0.179 0.225±0.058	 0.657±0.128 0.219±0.039	 0.793±0.260 ↑**17%** 0.269±0.092 ↑**17%**
Brain ●absolute ●relative	 2.011±0.105 0.681±0.040	 1.961±0.083 0.660±0.034	 1.970±0.096 0.658±0.047	 1.959±0.072 0.663±0.041
Pituitary ●absolute ●relative	 0.0164±0.0022 0.0055±0.0007	 0.0159±0.0018 0.0053±0.0006	 0.0163±0.0022 0.0054±0.0005	 0.0161±0.0020 0.0054±0.0006

TABLE 14. Organ Weights[a] P1 Main Study Rats				
Organ	Dose group (ppm)			
	Control	100	300	600/800
Adrenal				
•absolute	0.081±0.009	0.082±0.011	0.082±0.011	0.081±0.008
•relative	0.028±0.003	0.027±0.004	0.027±0.004	0.027±0.002

[a] Data obtained from Tables 51-52, page 284-289 in the study report. * α = 0.05; mean ±SD

Table 15. Historical control data for male reproductive and sex accessory gland weights	
Parameter	Historical range [A]
Absolute prostate (g)	1.115-1.392
Relative prostate (g/100 g BW)	0.181-0.235
Absolute testes (g)	3.439-3.936
Relative testes (g/100 g BW)	0.571-0.682
Absolute seminal vesicles (g)	1.558-2.109
Relative seminal vesicles (g/100 g BW)	0.266-0.356
Absolute epididymides (g)	1.335-1.545
Relative epididymides (g/100 g BW)	0.215-0.277

Data from Text Table 50, page 165 of the study report;
[A] mean ranges from 8 two-generation reproduction studies (2003-2008)

9. Histopathological Observations – P1 Males and Main Study Females

Microscopic examination: P1 males: Consistent with the kidney weight increases observed in P1 males at 800 ppm, treatment-related kidney histopathology findings were also observed at 800 ppm (Table 16). The lesion was described as a degenerative lesion involving the proximal convoluted tubules in the outer strip of the outer zone of the medulla, multifocal in distribution, and slight in degree. This lesion was primarily characterized by tubular epithelial cells, which were basophilic staining and had nuclei that were crowded together due to a decrease in the amount of cytoplasm (eosinophilic staining). Additionally, pyknotic nuclei were occasionally noted in these tubules. The remaining portions of these tubular profiles were considered normal. Affected tubules also had focally thickened basement membranes, adjacent interstitial fibrous connective tissue proliferation, and a mononuclear inflammatory cell infiltrate. **P1 Main Study Females:** There were no histopathological observations attributed to 2, 4-D exposure in the P1 females. Seven main study P1 females were non-pregnant (1 at 0 ppm, 3 at 300 ppm, and 3 at 600 ppm). For 3 of the 7 females, the reason for the failure to become pregnant could be attributed to effects on their mating partners (e.g., testicular atrophy, decreased spermatic elements, or prostate inflammation). There were no histopathological changes in the remaining females that explained their failure to mate or conceive. The terminal stage of estrous of the control and 600 ppm females was determined by histopathological examination of the vagina and uterus. There were no alterations in estrous cycle patterns in these rats. With the exception of preselected control and high-dose rats and the females that failed to mate or deliver litters, the remaining P1 females were not examined because of the lack of treatment-related effects in the subset of rats that were examined.

TABLE 16. Terminal Stage of Estrous (females)[B] and Microscopic Kidney Findings (both sexes)[a]				
Observation	**Dose group (ppm)**			
	Control	**100**	**300**	**600/800**
P1				
MALES				
Degeneration, proximal convoluted tubule, outer stripe, outer zone n=	10	10	10	11
● focal, very slight	0	4	1	0
● multifocal, very slight	9	4	6	3
● multifocal, slight	1	0	1	**8**
FEMALES				
Terminal stage of estrous n=	28	-	-	27
● estrus	0	-	-	1
● diestrus	6	-	-	7
● metestrus	3	-	-	5
● proestrus	19	-	-	14
Degeneration, proximal convoluted tubule, outer stripe, outer zone n=	10	0	0	10
● focal, very slight	0	-	-	0
● multifocal, very slight	0	-	-	0
● multifocal, slight	0	-	-	0

[a] Data obtained from Text Table 16, page 99 in the study report. [B] determined by histopathological exam of vagina and uterus, data from Appendix Table 39 (pages 750-751 of study report

10. Reproductive function - P1 Males and Main Study Females

a. Estrous cycle length and periodicity: No significant difference was observed in mean estrous cycle length in P1 females at any dose level compared to the control (Table 17). There was no indication of persistent estrus; i.e., greater than 2 consecutive days in estrus, and no apparent difference in the percentage of time spent in estrus or diestrus (see Figure 13 from study report in Appendix B).

TABLE 17. Estrous cycle data[a] P1 Main Study and Satellite Females				
Mean days per cycle	**Dose group**			
	Control	**100 ppm**	**300 ppm**	**600 ppm**
3.8	1	-	-	-
4.0	31	34	32	33
4.3	2	2	3	3
4.5	-	2	-	-
4.7	3	-	1	-
4.8	-	-	1	-
5.0	2	-	2	3
5.3	-	1	-	-
Mean±sd	4.1±0.3	4.1±0.2	4.1±0.3	4.1±0.3

[a] Data obtained from pages 688-695 in the study report; n=39

b. Sperm measures: P1 males: Sperm Motility: There were no significant, treatment-related, effects on sperm motility or progressive motility (Table 18). Although sperm motility was slightly lower in the 300 and 800 ppm males (95% vs. 98% in controls), the magnitude of the effect was minimal and could be attributed to a single outlier in each of the dose groups,. Removal of these two outlier values generated mean sperm motility values that were the same as the control (98%). Sperm/Spermatid Counts: With respect to testicular spermatid and epididymal sperm counts between control and 800 ppm males, there were no significant,

treatment-related, differences (Table 18). Sperm/spermatid counts were not conducted in the 100 ppm and 300 ppm groups due to the lack of effect at 800 ppm. Sperm Morphology: The proportion of abnormal sperm was not significantly different between control and 800 ppm males (Table 18). The slight increase in abnormal sperm at the 800 ppm dose level was due to a single outlier. When this one outlier was removed from the data, both the control and high-dose males had 1.4% abnormal sperm. Sperm morphology was not examined in the 100 ppm and 300 ppm dose groups due to the lack of effect at 800 ppm.

TABLE 18. Sperm data[a] (P1 males)

Parameter	Dose group			
	Control	100 ppm	300 ppm	800 ppm
Motile (%)	97.9±1.4	97.7±1.5	94.6±19.0	94.7±19.0
range[C]	93-100	94-100	94-100	96-100
Progressively Motile (%)	89.0±3.9	86.9±3.5	85.2±17.3	85.4±17.4
Range	80-95	81-93	82-94	83-96
Testicular spermatid counts				
Total Sperm (10^{06})	222.8±62.3	-	-	238.9±62.9
range[C]	98.8-365.4	-	-	67.4-393.7
Conc/g (10^{06})	119.7±30.0	-	-	135.1±30.7
range[C]	50.5-179.9	-	-	72.7-217.9
Epididymal sperm counts				
Total Sperm (10^{06})	235.3±70.8	-	-	226.3±77.5
range[C]	112.9-445.4			116.1-382.7[B]
Conc/g (10^{06})	669.2±186.6	-	-	681.1±207.3
range[C]	370.3-1113.6			441.3-1060.1 [B]
Proportion of abnormal sperm summary				
Abnormal sperm/total	0.014±0.015	-	-	0.020±0.036[A]

[a] Data obtained from pages 290-293 in the study report; n=27, except [A] (n=26); [B] one male with no sperm; [C] ranges from Appendix Tables 42-44, pages 778-783 of the study report

11. Reproductive Performance: There were no significant effects on any of the reproductive indices in the P1 rats at any dose level. Male and female mating, conception, fertility, and gestation indices and the percent post-implantation loss were comparable among the groups. Fertility index was decreased slightly (88.9%) at the mid- and high-dose levels, compared to the concurrent control (96.3%) but was within the range of historical control data (fertility male: 84-100%/female: 85.2-100%). Time-to-mating and gestation length were comparable among the groups. Results are summarized from the report in Table 19.

TABLE 19. Reproductive Data [a] Main Study P1 rats

Observation	Dose group (ppm)			
	Control	100	300	600
P1				
Number of males/females	27/27	27/27	27/27	27/27
Mean days per cycle (premating) [B]	4.13±0.32	4.09±0.27	4.15±0.32	4.10±0.27
Time to mating (days)	3.0±2.4	2.6±1.0	2.6±1.5	2.6±0.9
Mating index (%)	100	100	96.3	92.6
Conception index (%)	96.3	100	92.3	96
Fertility index (%)	96.3	100	88.9	88.9
Gestation index (%)	100	100	100	100
Gestation length (days)	21.7±0.5	21.4±0.5	21.6±0.5	21.5±0.5

[a] Data obtained from Table 38 (pages 270-271) and Appendix Table 20 (pages 688-695) in the study report; [B]calculated by reviewer (report combined Satellite GD 17 dams in their calculation)

B. GD 17 SATELLITE FEMALES

1. **Reproductive Indices and Litter Data:** Time to mating was comparable among the groups, although one 300 ppm GD 17 satellite dam was an outlier (14 days to mate). Post-implantation loss was slightly increased at 600 ppm compared to the control, although the standard deviation exceeded the mean in all cases. The mean number of fetuses per litter was comparable among the groups (Table 20).

TABLE 20. Reproductive Data [a] Satellite Females (GD 17)

Observation	Dose group (ppm)			
	0	100	300	600
P1 Satellite Females				
Mating index (%)	100	100	100	100
Conception index (%)	100	91.7	83.3	100
Fertility index (%)	100	91.7	83.3	100
Number of pregnant females	11	10	10	12
Number of corpora lutea (mean±SD)	16.9±1.3	17.4±2.5	15.8±1.4	16.3±1.8
Number of implantations (mean±SD)	16.6±1.5	16.4±1.6	15.5±1.2	15.8±1.7
Number of resorptions (mean±SD) Resorptions/litters w/ resorptions	0.9±1.1 2.2 (13/6) [B1]	0.8±1.1 2.0 (8/4)	0.5±0.7 1.3 (5/4)	1.5±2.2 2.0 (14/7) [B1]
Mean days per cycle (premating)	4.08±0.21	4.04±0.14	4.03±0.09	4.11±0.29
Time to mating (days)	2.7±1.0	2.2±1.0	2.9±3.6	2.9±1.0
Preimplantation loss (%)	1.68±2.88	4.81±9.44	1.77±2.85	2.29±6.46
Postimplantation loss (%)	5.54±6.94	4.79±6.93	3.31±4.59	**9.22±12.67**
Total number of fetuses	167	156	150	178
[B2]Fetuses/litter (mean±SD)	15.2±2.1 [B2]	15.6±1.8	15.0±1.5	14.8±2.5 [B2]
Total number of resorptions	13 E	7E, 1L	5E	14E

[a] Data obtained from Table 68 (page 318-319), Appendix Tables 20 (pages 688-695), 36 (pages 740-741), and 155 (pages 1426-1471) in the study report; [B]Table 68 lists: ([B1]2.0 (10/5) for control and 2.3 (18/8) for 600 ppm groups) and ([B2]15.7±2.0 for the control and 14.3±2.4 for 600 ppm groups); one 100 ppm female and two 300 ppm females were not pregnant; E = early resorption; L = late resorption;

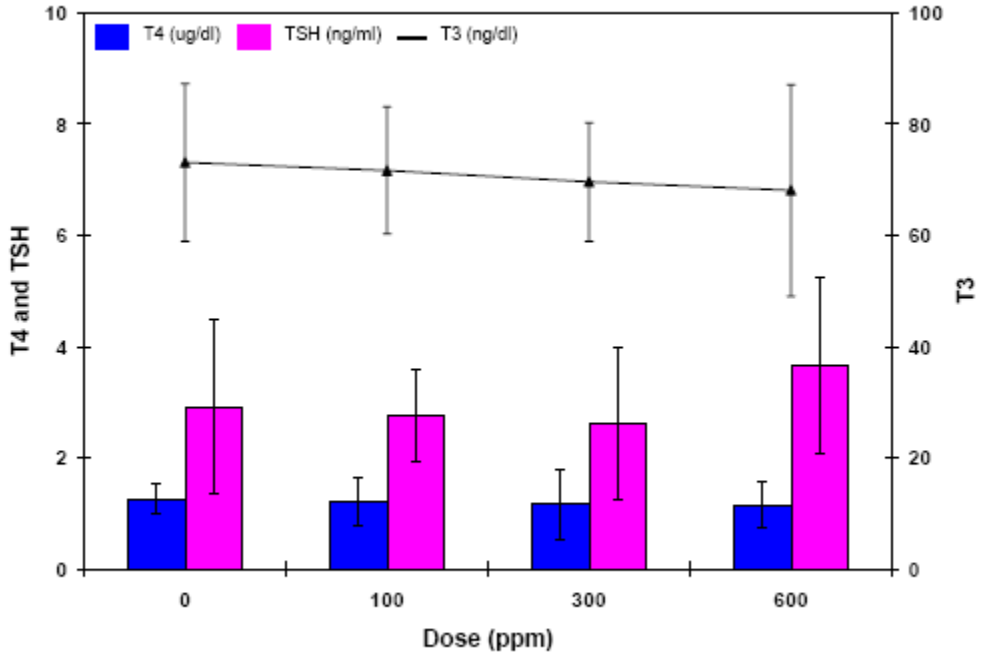
2. **Thyroid Hormones –GD 17 Satellite Females:** There were no statistically significant, treatment-related, differences in serum T3, T4, or TSH in the GD 17 satellite females (Table 21 and Figure 14, reproduced from page 203 of the study report). However, it is to be noted that the 600 ppm females displayed the predicted pattern of thyroid hormone changes that could signify a thyroid effect; i.e., ↓ T_3 and T_4 with ↑TSH levels, and the thyroid was examined microscopically in the P1 GD 17 satellite females (see below). These changes in the thyroid hormones along with the histopathological findings were considered treatment-related, although not adverse.

Table 21. Thyroid Hormone – GD 17 Satellite Females

Parameter	0 ppm	100 ppm	300 ppm	600 ppm
Females				
N=	11	10	10	12
T3 (ng/dL)	73.12±14.17	71.69±11.42	69.64±10.67 ↓**5%**	68.12±19.04 ↓**7%**
Range	52.83-95.78	53.99-93.33	47.94-89.35	35.95-104.12
T4 (µg/dL)	1.26±0.28	1.22±0.43	1.16±0.64 ↓**8%**	1.15±0.42 ↓**9%**
Range	0.82-1.66	0.77-1.42	0.46-2.17	0.61-1.87
TSH (ng/mL)	2.92±1.56	2.76±0.84	2.60±1.37	3.65±1.59 ↑**25%**
range **#>4**	1.03-5.62 2	1.47-4.02 1	1.17-3.45 (6.01)[A] 1 [A]	1.25-6.23[B] 5

Data from Table 59, page 305 of study report; [A] identified as outlier in report; [B] not identified as outlier in report

FIGURE 14. Thyroid Hormones – GD 17



3. **Hematology Parameters – P1 Satellite Females:** No treatment-related changes in hematology parameters, including differential white blood cell counts and prothrombin time, were reported. Table 22 provides the platelet count findings since F1 offspring Set 1a males displayed a reduction in platelet counts (↓14%) on PND 70.

Table 22. – P1 Satellite Females				
Parameter	**0 ppm**	**100 ppm**	**300 ppm**	**600 ppm**
Females				
N=	11	10	10	12
Platelet count (E3/UL)	1246±165	1228±148	1248±148	1199±248 ↓4%

Data from Tables 60 and 62, pages 306 and 308 of study report

4. **Clinical Chemistry– P1 Satellite Females:** Clinical chemistry parameters were comparable among the groups of pregnant females.

5. **Survival – P1 Satellite Females:** All P1 satellite females survived to scheduled necropsy.

6. **Organ Weights P1 Satellite Females on GD 17:** Terminal body weights were comparable among the groups. The thyroid was the only organ weighed in the P1 satellite females. Although there were minor differences in thyroid weights, there was no dose-response (Table 23).

Table 23. – P1 Satellite Females Terminal Body and Organ Weights				
	0 ppm	**100 ppm**	**300 ppm**	**600 ppm**
Females				
n=	11	10	10	12
Body Weight (g)	398.2±14.6	388.0±19.2	383.9±25.7	397.3±17.5
Thyroid				
Absolute (g)	0.0172±0.0022	0.0188±0.0038 ↑**9%**	0.0167±0.0027	0.0187±0.0026 ↑**9%**
Relative (g/100)	0.0043±0.0006	0.0048±0.0008	0.0043±0.0006	0.0047±0.0006

Data from Table 65, page 311 of study report

7. **Gross Pathology Observations – P1 Satellite Females:** There were no gross findings attributed to treatment in the P1 satellite females. None of the non-pregnant females (#4439 at 100 ppm and #4478 and #4484 at 300 ppm) showed gross findings associated with the failure to conceive.

8. **Histopathological Observations – P1 Satellite Females:** Although changes in thyroid hormones were not significant, the pattern of thyroid changes at the high dose was consistent with a potential perturbation in thyroid function (i.e., decreased T4 [9%] and T3 [7%] with increased TSH [25%]); therefore, thyroid glands from GD 17 satellite females were examined histopathologically. Three of 12 satellite females at 600 ppm had follicles that were smaller than those of the controls, and the colloid within the smaller follicles frequently contained clear vacuoles along the periphery of the follicular epithelial cells suggestive of colloid resorption. This change was graded as very slight. There were no adverse pathologic observations (i.e., degeneration, necrosis etc.) associated with the smaller follicles, suggesting that these alterations were adaptive rather than pathologic and consistent with a more rapid colloid turnover. Consequently, these thyroid alterations were considered treatment-related but not adverse. There were no effects on thyroid histopathology at 100 ppm or 300 ppm, consistent with the lack of thyroid hormone changes at these dose levels (Table 24).

TABLE 24. Microscopic Thyroid Findings[a] P1 Satellite Females				
Observation	**Dose group (ppm)**			
	Control	**100**	**300**	**600**
P1 Satellite Females				
Number of dams examined	11	11	12	12
Cyst; with keratinous debris; focal/multifocal; very slight	8	8	7	4
Aggregates of mononuclear cells, multifocal, very slight	0	0	0	1
Decreased follicle size, multifocal, very slight	0	0	0	3

[a] Data obtained from Appendix Table 155 pages 1426-1471 in the study report.

C. OFFSPRING: Main Study F1

1. **Viability and clinical signs**: The following observations were reported: There was no treatment-related effect on the number of live F1 pups born/litter (or the number of dead pups) or on pup survival or sex ratio (Table 25). There was no mention of clinical observations of the offspring during lactation. The number dying initially (PND 0-4) was slightly greater at 600 ppm than in the other groups.

TABLE 25. Litter Data [a] Main Study (F1 litters)				
Observation	**Dose group (ppm)**			
	Control	**100**	**300**	**600**
Number of litters	27	28	24	24
Number of implantations	407	423	373	374
Implantations/litter	15.7	15.1	15.5	15.6
Gestation survival index (%)	98.6	99.5	98.5	99.7
Number of fetuses born	369	376	325	344
Number born live (mean±SD)	364 (13.5±2.7)	374 (13.4±3.3)	320 (13.3±2.1)	343 (14.3±2.6)
Number born dead (mean±SD)	5 (0.2±0.8)	2 (0.1±0.4)	5 (0.2±0.7)	1 (0.0±0.2)
# Deaths days 0-4 (% of live born)	5 (1.37)	4 (1.07)	5 (1.56)	8 (2.33)
Mean litter size Day 1	13.3±2.6	13.2±3.4	13.1±1.9	14.1±2.6
Mean litter size Day 4 [b]	13.3±2.6	13.1±3.3	13.1±1.9	14.0±2.5
Mean litter size Day 4 [c]	9.8±1.2	9.5±1.5	10.0±0.2	9.9±0.9
Mean litter size Day 7	9.8±1.2	9.5±1.5	9.9±0.3	9.8±0.8
Mean litter size Day 14	9.7±1.2	9.5±1.5	9.9±0.3	9.8±0.8
Mean litter size Day 21	9.7±1.2	9.4±1.5	9.9±0.3	9.8±0.8
Total number of resorptions[d]	-	-	-	-
Postimplantation loss (%)	10.03±9.91	12.17±15.39	13.63±12.34	8.64±12.14
Sex ratio (M:F; day 1)	54:46	47:53	50:50	55:45
Survival				
Day 1	98.6	98.7	98.4	98.5
Day 4	98.6	98.4	98.4	97.7
Day 7	100	99.3	99.6	99.6
Day 21	99.6	98.9	99.2	99.2

[a] Data obtained from Tables 38 (pages 270-271), 69 (page 320) in the study report;
[d] could not determine (data not provided); Gestation index (% newborn pups alive at birth); b Before standardization (culling); c After standardization

2. **Body weight (F1 offspring – Main Study):** Litter weight data were not provided. On PND 1, male pups (↓3%) and female pups (↓4%) in the 600 ppm group weighed slightly less than control pups (not statistically significant), which may be the result of the slightly larger litter sizes in the 600 ppm group (14.1 pups/litter at 600 ppm and 13.3 pups/litter at 0 ppm). By PND 4, pre-culling body weights in 600 ppm pups were 8% lower than controls and this decrease was

sustained on PND 7 (Table 26). These decreases in pup body weight were not statistically significant, but coincided with a statistically significant decrease in feed consumption in the dams prior to adjustments of dietary concentrations and lower body weights/gains in 600 ppm dams on PND 1-4 and PND 4-7. At time points after dietary concentration adjustment (PND 14 and PND 21), 600 ppm dams had body weights and feed consumption values similar to control dams. Pup body weights in the 600 ppm group remained ↓5%-6% below control values during the PND 14-21 interval likely related to combined 2, 4-D intake through diet and milk. Pup body weights were comparable to the control at 100 ppm and 300 ppm during lactation.

TABLE 26. Mean (±SD) F1 Pup Body Weights (g) [a] (litter means)

Dose group (ppm)								
Lactation Day	**0**	**100**	**300**	**800**	**0**	**100**	**300**	**600**
	F_1 Pups – male				**F_1 Pups – female**			
1	7.3±0.6	7.2±0.8	7.4±0.7	7.1±0.7 ↓3%	7.0±0.7	6.9±0.8	6.9±0.6	6.7±0.6 ↓4%
4 b	10.4±1.1	10.2±1.6	10.4±1.0	9.6±1.2	10.0±1.3	9.8±1.5	9.8±1.0	9.2±1.0
4 c	10.4±1.1	10.3±1.7	10.4±1.0	9.7±1.2	10.0±1.3	9.8±1.5	9.8±1.0	9.1±1.0
7	16.6±1.4	16.5±2.3	16.4±1.4	15.5±1.8↓7%	15.9±1.6	15.8±2.1	15.6±1.4	14.7±1.4 ↓8%
21	52.5±4.5	51.4±5.4	50.3±4.2	49.4±5.4↓6%	50.6±4.7	49.6±5.3	48.7±4.1	47.6±4.0 ↓6%

[a] Data from Table 70, page 321 in the study report; n = 27 (control), 28 (100 ppm), and 24 (300 ppm and 800 ppm/600 ppm)
[b] Before standardization (culling); [c] After standardization (culling)

3. **Body Weight F1 Weanlings - PND 21 and PND 22:** Because of assignment of pups to different groups on PND 21, multiple sets of pup body weights were collected during the PND 21-22 interval. Pups were assigned to the groups based on day of birth (not body weight), so no stratification by body weight was possible. PND 22 male pups selected for organ weight measurements displayed a statistically significant (↓10%) decrease in body weight compared to control, while those selected for perfusion displayed a non-significant (↓6%) decrease (Table 27).

TABLE 27. Mean (±SD) F1 Weanlings - Pup Body Weights (g) [a] – PND 21 and PND 22								
Dose group (ppm)								
Group	**0**	**100**	**300**	**800**	**0**	**100**	**300**	**600**
	F_1 Pups – male				**F_1 Pups – female**			
PND 22 (n=10 pups/dose) Organ wts	57.9±3.8	52.2±3.8*	52.8±3.9	51.9±7.4*↓10%	52.7±6.1	50.4±7.9	50.5±4.4	51.2±5.9
PND 22 (n=12 pups/dose) Perfusions	57.1±3.9	56.4±5.7	52.5±4.8	53.7±6.1 ↓6%	54.1±5.4	53.1±4.5	49.9±3.8	50.0±4.3 ↓8%
PND 22 combined (n=22 pups/dose)	57.5±3.8	54.5±5.3	52.6±4.3*	52.8±6.6* ↓8%	-	-	-	-
PND 21 (24-28 litters/dose) all pups	52.5±4.5	51.4±5.4	50.3±4.2	49.4±5.4 ↓6%	50.6±4.7	49.6±5.3	48.7±4.1	47.6±4.0 ↓6%

[a] Data obtained from Text Tables 32 and 33, pages 146-147 in the study report. *α = 0.05

4. **Achieved Test Material Intake**: Test material intake (expressed as mg/kg/day) is shown in Table 28. For F1 offspring, doses were nearly twice the targeted dose levels of 5, 15 and 30 or 40 mg/kg/day in Sets 1a, 1b and 2a, all of which were euthanized by PND 70. As the F1 rats were maintained on test diet beyond PND 70, feed consumption decreased and TMI approached the targeted dose levels. Although closest to the target, TMI in Set 3 male and female F1 offspring were reported as still exceeding targeted dose levels by 37-56% across all dose levels.

TABLE 28. Mean Test Material Intake (TMI) for F1 Male and Female Offspring (mg/kg body weight/day) [a]						
	Male			**Female**		
	100 ppm	**300 ppm**	**800 ppm♪**	**100 ppm**	**300 ppm**	**600 ppm♪**
Set 1a PND 28-69	9.24±2.25	28.4±7.18	76.6±18.4	9.56±1.99	28.8±6.10	57.9±12.7
Set 1b PND 28-56	9.88±2.31	29.5±7.43	81.7±19.8	10.1±2.35	30.0±6.90	59.2±14.3
Set 2a PND 28-70	9.15±2.16	28.4±6.66	75.3±18.8	9.66±1.91	28.7±5.69	58.4±11.5
Set 2b PND 28-84	8.67±2.34	25.8±7.27	71.8±19.1	9.05±1.91	26.7±5.65	55.3±11.4
Set 3 PND 28-133	6.83±2.43	20.9±7.14	55.6±19.3	7.59±1.89	23.3±5.94	46.7±11.6

[a] Data (means only) obtained from page 137 in the study report (s.d. were obtained from each Set's TMI data tables by reviewer).
♪ did not receive adult dietary concentration until PND 35; TMI calculations corrected for adjusted diets given on PND 28-35

5. **Anogenital Distance – F1 Offspring:** There were no significant, treatment-related, differences in absolute or relative anogenital distance in either sex (Table 29).

Table 29. Anogenital Distance – F1 Offspring				
Parameter	**0 ppm**	**100 ppm**	**300 ppm**	**600/800 ppm**
Males				
AGD (mm)	3.92±0.27	3.78±0.38	3.83±0.23	3.74±0.28
Relative AGD [B]	2.02±0.13	1.95±0.16	1.97±0.11	1.95±0.12
Body weight (g)	7.3±0.6	7.2±0.8	7.4±0.7	7.1±0.7
Females				
AGD (mm)	2.08±0.25	2.03±0.24	2.06±0.21	2.01±0.27
Relative AGD [B]	1.09±0.13	1.06±0.11	1.08±0.12	1.06±0.13
Body weight (g)	7.0±0.7	6.9±0.8	6.9±0.6	6.7±0.6

Data from Table 71, page 322 of report; [B] mm AGD/cubed root of BW in grams; n=27 (control); 28 (100 ppm); 24 (300 ppm and 600/800 ppm)

6. **Nipple Retention:** There was no difference in nipple/areolae retention between the control and high-dose group in either sex (Table 30). Consequently, the lower dose levels were not assessed for this endpoint.

Table 30. Nipple/Areolae Retention				
parameter	**0 ppm**	**100 ppm**	**300 ppm**	**600/800 ppm**
Males				
# of nipples	0	NA	NA	0
Females				
# of nipples	12±0.1	12±0.0	12±0.1	12.0±0.1

Data from Table 72, page 323 of report; n=27 (control); 28 (100 ppm); 25 (300 ppm and 600/800 ppm)

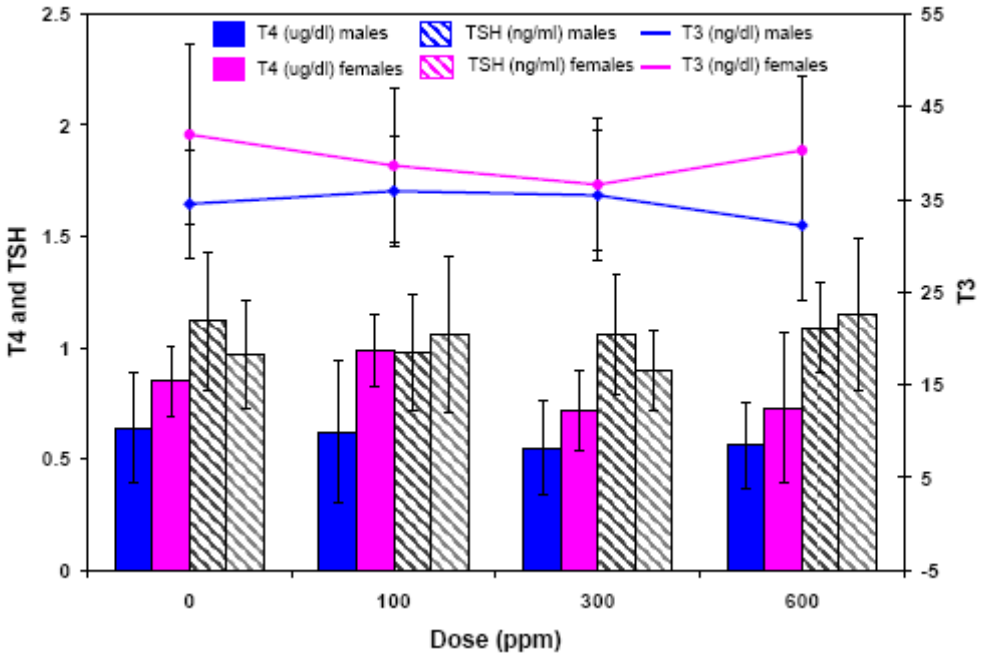
7. **Hormone Measurements - Thyroid**

a. **Thyroid Hormones - F1 offspring on PND 4:** There were no statistically-significant differences in serum T3, T4, or TSH in PND 4 culled pups (Table 31; Figure 15 reproduced from page 204 of the study report). T4 was reduced (↓12%) in both sexes at their high-dose level, and female PND 4 pups showed an increase in TSH (↑19%) at 600 ppm. However, a dose response was not evident for any of the alterations in these parameters

Table 31. Thyroid Hormone – F1 PND 4 (culled) pups				
parameter	**0 ppm**	**100 ppm**	**300 ppm**	**600/800 ppm**
Males				
T3 (ng/dL)	34.51±5.83 (9)	35.90±5.99 (8)	35.46±7.07 (8)	32.19±8.17 ↓**7%**
T4 (µg/dL)	0.64±0.25	0.62±0.32 (8)	0.55±0.21 ↓**14%**	0.56±0.19 ↓**12%**
TSH (ng/mL)	1.12±0.31	0.98±0.26 (7)	1.06±0.27 (9)	1.09±0.20
Females				
T3 (ng/dL)	41.99±9.78	38.64±8.28 ↓**8%**	36.59±7.15 (6) ↓**13%**	40.29±8.06 (9)
T4 (µg/dL)	0.85±0.16	0.99±0.16	0.72±0.32 (9) ↓**15%**	0.73±0.23 ↓**14%**
TSH (ng/mL)	0.97±0.24	1.06±0.35 ↑**9%**	0.90±0.18 (9)	1.15±0.34 ↑**19%**

Data from Tables 73 and 74, pages 324 and 325 of report; n=10, unless ( )

FIGURE 15. Thyroid Hormones – PND 4



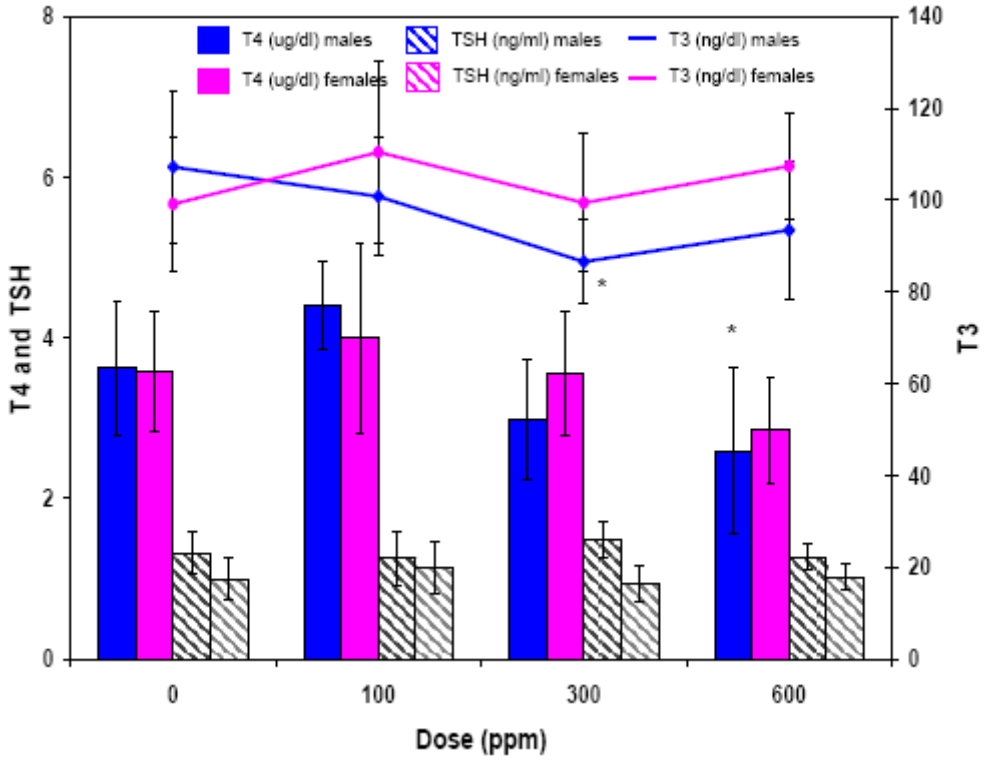
b. Thyroid Hormones - F1 offspring (PND 22 Weanlings): F1 PND 22 males displayed a statistically-significant reduction (↓28%) in T4 at 800 ppm, and F1 PND 22 females displayed a non-statistically significant reduction (↓20%) in T4 at 600 ppm. T3 was reduced in the males at 300 ppm (↓19%) and 800 ppm (↓13%), but there was no dose response (Table 32; Figure 16 reproduced below from page 205 of the study report).

Table 32. Thyroid Hormone – F1 PND 22 Weanlings

Parameter	0 ppm	100 ppm	300 ppm	600/800 ppm
Males				
T3 (ng/dL)	107.22±16.59	100.82±12.88	86.56±9.16* ↓**19%**	93.46±15.06 ↓**13%**
T4 (µg/dL)	3.62±0.84	4.40±0.54	2.98±0.75	2.59±1.04* ↓**28%**
TSH (ng/mL)	1.32±0.24	1.25±0.59	1.48±0.73 ↑**12%**	1.27±0.37
Females				
T3 (ng/dL)	99.14±14.68	110.43±20.01	99.42±15.02	107.42±11.68
T4 (µg/dL)	3.57±0.75	3.99±1.19	3.55±0.77	2.85±0.66 ↓**20%**
TSH (ng/mL)	0.99±0.26	1.13±0.33	0.94±0.22	1.02±0.16

Data from Tables 75 and 76, pages 326 and 327 of report; n=10; * α = 0.05
Males not given adult dietary concentration until PND 35.

FIGURE 16. Thyroid Hormones – PND 22



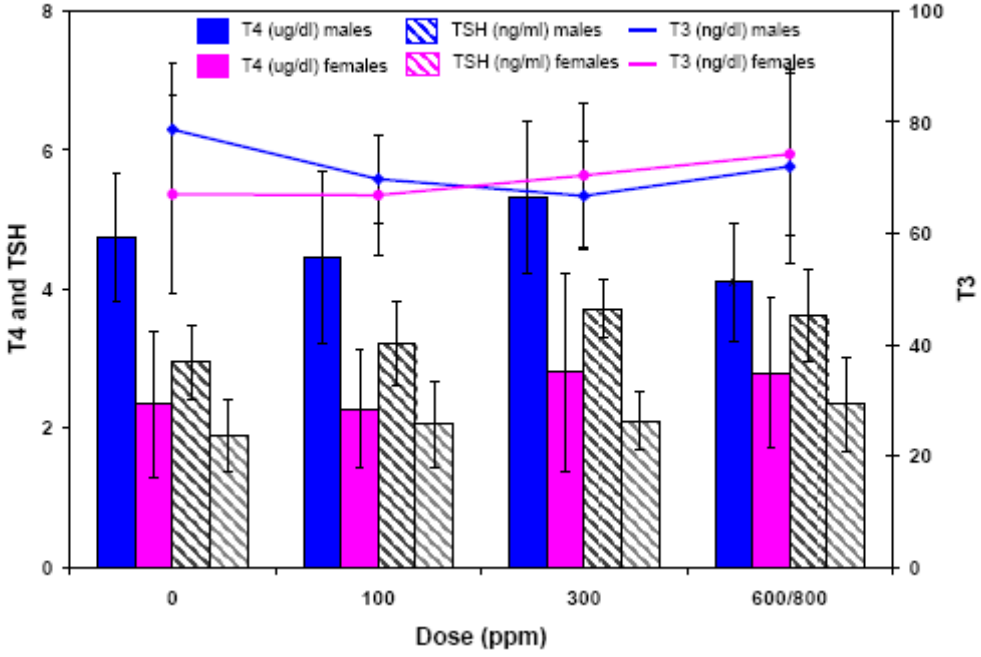
c. **Thyroid Hormones – Set 1a F1 Offspring (PND 62-64):** Both sexes displayed increased TSH at 300 ppm and at 800 ppm (males)/600 ppm (females), although the increase in males was not dose-related and none of the differences in thyroid hormone levels were statistically significant. T4 was decreased at 800 ppm in males (Table 33; Figure 17 reproduced below from page 207 of the study report).

Table 33. Thyroid Hormone – F1 Set 1a Males (PND 62-64)

Parameter	0 ppm	100 ppm	300 ppm	600/800 ppm
Males				
T3 (ng/dL) **range**	78.69±12.07 63.03-102.07	69.78±7.91 58.34-80.05	66.77±9.69 46.97-84.54	72.03±17.40 43.51-94.29
T4 (µg/dL) **range**	4.75±0.92 3.50-6.44	4.46±1.23 2.93-6.56	5.31±1.09 ↑**12%** 2.79-6.35	4.11±0.85 ↓**13%** 2.63-5.45
TSH (ng/mL) **range**	2.95±0.74 2.12-3.42 (4.73)[A]	3.21±1.29 1.91-5.66	3.72±0.97 ↑**26%** 2.77-5.26	3.62±1.20 ↑**23%** 2.11-5.77
Females				
T3 (ng/dL) **range**	67.08±17.71 51.49-81.31 (109.79) [A]	66.89±10.71 46.65-79.02	70.45±12.99 57.25-96.84	74.28±14.67 ↑**11%** 58.13-100.28
T4 (µg/dL) **range**	2.35±±1.05 1.00-4.72	2.27±0.85 0.99-3.36	2.80±1.43 ↑**19%** 1.54-5.65	2.79±1.08 ↑**19%** 1.29-5.03
TSH (ng/mL) **range**	1.89±0.53 0.93-2.60	2.05±0.61 1.24-3.00	2.10±0.42 ↑**11%** 1.84-2.46 (1.03) [A]	2.34±0.67 ↑**24%** 1.66-2.41 (4.15) [A]

Data from Tables 77 and 78, pages 328 and 329 of report; n=10; [A] outlier

FIGURE 17. Thyroid Hormones – PND 62-64



8. **Sexual maturation (F_1):** F1 males at 800 ppm displayed a slight delay in preputial separation (1.6 days), which was accompanied by a very slight reduction in body weight compared to the control (↓2.1 grams; 99% of control). This reduction is within the normal variability for this measure and is not considered to be toxicologically relevant or indicative of adverse effect on sexual maturation. There was no evidence of retained preputial threads. The age at vaginal opening was comparable among the groups of F1 females (Table 34).

Table 34. Puberty Onset – F1 Offspring (Sets 1, 2, 3)

Parameter	0 ppm	100 ppm	300 ppm	600/800 ppm
Preputial separation	**Males**			
Age (days)	43.0±1.3	43.6±1.5	43.3±1.5	44.6±2.3* (↑1.6 days)
Body weight (g)	223.2±13.7	230.3±14.9	221.0±15.7 (↓2.2 g)	221.1±12.3 (↓2.1 g)
#≥ 43.0	18	17	11	17
#≥ 44.6	3	5	7	11
Vaginal opening	**Females**			
Age (days)	32.0±0.9	31.9±0.7	31.8±1.0	32.3±0.8
Body weight (g)	108.8±6.3	106.5±6.3	106.0±7.7	104.1±9.9 (↓4.7 g)

Data from Tables 87 and 88, pages 353 and 354 of report; n=27 (control); 28♂/27♀ (100 ppm); 24 (300 ppm and 600/800 ppm); * α = 0.05

C. OFFSPRING: F1 Offspring Set 1a Males and Females (PND 70) – General Toxicity

1. **Survival:** There was no treatment-related effect on F1 Set 1a male or female survival. All offspring survived to scheduled necropsy (PND 70).

2. **Body weight (F1 Set 1a Males and Females): Males**: Males displayed significant decreases in body weight at 800 ppm (↓11%-17%) throughout the study period, with the magnitude of the reduction lessening with time of exposure (Table 35). Consistent with these body weight decrements, body weight gains were significantly decreased in 800 ppm males at all intervals

from PND 21-56 (↓11%-25%). Body weights/body weight gains were not significantly altered at 100 ppm and 300 ppm. **Female:** There were no treatment-related effects on body weights/body weight gains in females at any dose level.

TABLE 35. Mean (±SD) F1 Body Weights/Gains of Set 1a (PND 70) (g) [a]

Day	Dose group (ppm)							
	0	100	300	800	0	100	300	600
	F_1 Set 1a – male				F_1 Set 1a – female			
21	53.7±5.4	50.2±5.2	48.7±5.5	47.6±5.0 ↓11%	50.9±4.7	49.4±4.8	48.0±3.7	48.0±5.1
28	92.5±6.5	88.7±8.7	86.5±9.6	76.9±7.8* ↓17%	85.1±5.7	83.6±4.4	81.9±5.9	79.3±6.9 ↓7%
69	412.8±28.1	403.4±38.7	390.7±46.5	366.3±25.2* ↓11%	247.8±21.5	243.2±13.6	246.9±22.4	243.7±22.5
21-28	38.9±3.5	38.5±4.1	37.8±5.7	29.3±6.6* ↓25%	34.2±3.3	34.2±3.0	33.9±2.8	31.2±2.8 ↓9%
21-69	359.2±30.0	353.3±35.6	343.3±45.0	318.8±22.6 ↓11%	197.0±18.9	193.6±16.8	198.8±22.0	195.6±21.2

[a] Data obtained from Tables 91-92, pages 357-360 in the study report. n=10, except day 69 [female 100 ppm (n=8); female 300 ppm (n=9)] due to BW inadvertently not collected

3. **Hematology**: Platelet counts were reduced (↓14%) in the 800 ppm males compared to the control males, with all of the 800 ppm values being less than the mean control value (Table 36). A similar reduction was not observed in the F1 Set 1a females. There were no other changes in the hematological parameters monitored, and the differential white blood cell counts and prothrombin times were similar among the groups (both sexes).

Table 36. Platelet Counts – F1 Offspring (Set 1a)

Parameter	0 ppm	100 ppm	300 ppm	600/800 ppm
Males				
Platelet counts (E3/UL)	1441±234	1386±181	1354±172	1236±151 ↓**14%**
Range	1165-1908	1147-1711	1089-1629	1015-1426
Females				
Platelet counts (E3/UL)	1365±182	1361±156	1388±166	1341±204

Data from Tables 97 and 98, pages 365 and 366 of report; n=10

4. **Clinical Chemistry: F1 Males**: In the Set 1a 800 ppm males, there was a slight, but statistically significant, increase (18%) in ALT levels (Table 37) but a lack of corresponding liver histopathology (see below). Triglyceride levels were increased (↑23% and 31%) at 300 ppm and 800 ppm, respectively, compared to the control. No significant, treatment-related, changes in glucose or electrolyte levels were reported. **F1 Females**: Increased ALT ((↑25%) and triglycerides ((↑43%) values were observed in females at 600 ppm compared to the control (Table 36). No significant, treatment-related changes in glucose or electrolyte levels were noted.

Table 37. Clinical Chemistry – F1 Offspring (Set 1a) – PND 70

Parameter	0 ppm	100 ppm	300 ppm	600/800 ppm
Males				
ALT (U/L)	40±5	37±5	42±7	47±7* ↑**18%**
Triglycerides (mg/dL)	39±14	41±12	48±17 ↑**23%**	51±14 ↑**31%**
Females				
ALT (U/L)	32±7	34±7	36±5	40±9 ↑**25%**
Triglycerides (mg/dL)	40±12	34±5	41±11	57±24 ↑**43%**

Data from Tables 103 and 104, pages 371 and 372 of report; n=10, except 100 ppm females (n=9)

5. **Urinalysis: PND 64-66**: There were no treatment-related changes in urinalysis parameters at any dose level. The females at 300 ppm and 600 ppm displayed higher urine volumes (not dose-related) and lower specific gravity but these minor differences, which were not present in the males, were considered to be due to normal variation and not treatment related.

6. **Organ and Organ/Body Weights Summary (F1 Set 1a Males and Females, PND 70): Males:** Terminal body weights were significantly decreased by 10% in males at 800 ppm, but were not significantly affected at 100 ppm or 300 ppm. Decreased absolute and related spleen weights were observed at all dose level, with the 800 ppm dose group displaying a 17% deficit (not statistically significant) compared to the control. Slight, but significantly, decreased absolute liver (↓16%) weight, relative liver weight (↓ 6%, not significant), absolute brain (↓4%) weight, and increased relative brain weight (↑7%, not significant) were observed at 800 ppm. Additionally, absolute thyroid weight was decreased (↓11%) and pituitary weight was deceased (absolute ↓14%/relative ↓6%) in males at 800 ppm, although statistical significance was not attained. Other decreases in absolute organ weights at 800 ppm may be attributed to the 10% lower body weight at 800 ppm (Table 38). Organ weights were not significantly altered at 100 ppm and 300 ppm. **Females:** Terminal body weights were not significantly affected. At 600 ppm, uterine weights (absolute ↑31%; relative ↑32%) were increased and thymus weights were decreased (absolute ↓12%; relative ↓10%) compared to the control, although statistical significance was not attained. Relative kidney weights were significantly increased (↑11%) at 300 and 600 ppm, which was similar to the increase in kidney weights observed in females in the 2,4-D range-finding study (5% and 16% increase in relative kidney weights at 400 and 800 ppm, respectively; MRID 47417901). Non-significant increases in absolute kidney weights also were seen (11% and 9% at 300 and 600 ppm, respectively). Kidney weights were not significantly altered in females at 100 ppm. These kidney findings were considered to be treatment-related but were not considered adverse in the absence of corresponding histopathological findings. It is to be noted that the kidney appears to be a target organ and histopathology was observed in the kidney in other sets. There were no significant, treatment-related effects on absolute or relative adrenal, heart, liver, brain, spleen, pituitary, or thyroid gland weights at any dose level in the Set 1a females (Table 38).

TABLE 38. Organ Weights[a] F1 Set 1a Offspring – General Toxicity (PND 70)

Organ	Dose group (ppm)			
	Control	100	300	600/800
F1 Set 1a MALES (PND 70)				
Terminal Body Weight (g) N=	379.4±24.4 10	373.21±31.6 10	379.3±52.1 10	340.1±22.7* ↓**10%** 10
Kidneys ●absolute ●relative	 3.108±0.302 0.819±0.064	 3.004±0.395 0.803±0.056	 3.208±0.412 0.850±0.075	 3.050±0.334 0.899±0.102
Liver ●absolute ●relative	 12.076±1.343 3.177±0.202	 11.538±1.076 3.090±0.059	 12.353±2.269 3.244±0.185	 10.169±0.809* ↓**16%** 2.994±0.191 ↓6%
Thyroid ●absolute ●relative	 0.0195±0.0031 0.0051±0.0008	 0.0204±0.0028 0.0055±0.0007	 0.0190±0.0035 0.0050±0.0009	 0.0174±0.0019 ↓**11%** 0.0052±0.008
Testes ●absolute ●relative	 3.306±0.115 0.875±0.074	 3.366±0.239 0.904±0.060	 3.201±0.358 0.849±0.077	 3.237±0.277 0.954±0.088
Prostate ●absolute ●relative	 0.753±0.139 0.198±0.035	 0.776±0.164 0.207±0.035	 0.791±0.129 0.209±0.021	 0.707±0.144 ↓**6%** 0.208±0.043
Seminal vesicles w/cg				

TABLE 38. Organ Weights[a] F1 Set 1a Offspring – General Toxicity (PND 70)				
Organ	**Dose group (ppm)**			
	Control	**100**	**300**	**600/800**
●absolute	1.072±0.180	1.147±0.196	1.141±0.218	1.010±0.163
●relative	0.282±0.041	0.307±0.042	0.301±0.045	0.298±0.054
Epididymides				
●absolute	0.895±0.063	0.862±0.053	0.870±0.085	0.842±0.062 ↓**6%**
●relative	0.237±0.028	0.232±0.014	0.232±0.027	0.248±0.021
Brain				
●absolute	2.008±0.076	1.978±0.089	2.023±0.054	1.927±0.035* ↓**4%**
●relative	0.532±0.047	0.532±0.035	0.541±0.068	0.569±0.035
Pituitary				
●absolute	0.0116±0.0010	0.0114±0.0013	0.0109±0.0019	0.0100±0.0012 ↓**14%**
●relative	0.0031±0.0003	0.0031±0.0003	0.0029±0.0004	0.0029±0.0002
Thymus				
●absolute	0.545±0.080	0.561±0.099	0.599±0.074	0.525±0.070 ↓**4%**
●relative	0.144±0.024	0.150±0.022	0.160±0.022	0.155±0.023
Adrenals				
●absolute	0.064±0.013	0.066±0.011	0.061±0.011	0.056±0.009 ↓**12%**
●relative	0.017±0.004	0.018±0.004	0.016±0.003	0.016±0.003
Spleen				
●absolute	0.766±0.194	0.714±0.098	0.700±0.082	0.635±0.071 ↓**12%**
●relative	0.201±0.044	0.192±0.026	0.186±0.023	0.187±0.020 ↓**7%**
F1 Set 1a Females (PND 70)				
Terminal Body Weight (g)	229.6±21.1	228.3±13.4	230.9±21.6	224.6±19.0
N=	10	10	10	10
Kidneys				
●absolute	1.749±0.162	1.812±0.173	1.946±0.108 ↑**11%**	1.905±0.272 ↑**9%**
●relative	0.765±0.078	0.793±0.041	0.846±0.053* ↑**11%**	0.846±0.068* ↑**11%**
Liver				
●absolute	7.226±0.606	7.259±0.767	7.194±0.842	7.091±0.760
●relative	3.152±0.132	3.179±0.258	3.114±0.186	3.154±0.133
Thyroid				
●absolute	0.0159±0.0012	0.0157±0.0026	0.0144±0.0018	0.0151±0.0024
●relative	0.0070±0.0007	0.0069±0.0010	0.0063±0.0012	0.0067±0.0007
Ovaries				
●absolute	0.099±0.018	0.105±0.022	0.105±0.018	0.108±0.014 ↑**9%**
●relative	0.044±0.011	0.046±0.010	0.045±0.007	0.048±0.007
Uterus				
●absolute	0.5229±0.1082	0.5354±0.1114	0.5521±0.1540	0.6861±0.2367 ↑**31%**
●relative	0.2307±0.0596	0.2351±0.0491	0.2436±0.0840	0.3039±0.1003 ↑**32%**
Brain				
●absolute	1.887±0.072	1.850±0.076	1.859±0.068	1.864±0.083
●relative	0.826±0.057	0.812±0.037	0.812±0.086	0.835±0.079
Pituitary				
●absolute	0.0116±0.0011	0.0125±0.0022	0.0116±0.0012	0.0126±0.0024 ↑9%
●relative	0.0051±0.0007	0.0055±0.0009	0.0050±0.0004	0.0056±0.0007
Adrenals				
●absolute	0.063±0.006	0.066±0.011	0.067±0.010	0.063±0.010
●relative	0.028±0.004	0.029±0.004	0.029±0.004	0.028±0.004
Thymus				
●absolute	0.522±0.109	0.490±0.116	0.508±0.133	0.460±0.104 ↓**12%**
●relative	0.227±0.042	0.214±0.045	0.220±0.053	0.204±0.036 ↓**10%**
Spleen				
●absolute	0.493±0.081	0.489±0.082	0.508±0.074	0.465±0.104 ↓**7%**
●relative	0.215±0.027	0.213±0.029	0.220±0.025	0.206±0.033 ↓4%

[a] Data obtained from Tables 111-112, page 379-384 in the study report. * α = 0.05; mean ±SD

7. **Gross Pathology Observations (F1 Set 1a Males and Females):** There were no gross observations attributed to dietary 2, 4-D exposure in the F1 Set 1a rats.

8. **Histopathological Observations (F1 Set 1a Males and Females): Males**: The kidney appears to be a target organ. There was a dose-related increase in microscopic findings in the kidney in males. A degenerative lesion involving the proximal convoluted tubules in the outer stripe of the outer zone of the medulla, which was multifocal in distribution and very slight or slight in degree, was observed in the kidney in males at the 300 ppm and 800 ppm dose levels (Table 39). There were no histopathological alterations in the kidneys at 100 ppm. With the exception of kidneys, other tissues from animals in the low- and middle-dose groups were not examined, because of the lack of treatment-related effects in the high-dose animals. **Females:** At 600 ppm, females displayed microscopic, treatment-related, kidney effects similar in nature to those observed in the males; however, these effects were very slight in degree (Table 39). There were no histopathological alterations in the kidneys at 100 ppm or 300 ppm. Regarding the terminal stage of estrous, 2 of 10 females at 300 ppm and 3 of 10 females at 600 ppm displayed proestrus whereas none of the 10 females in the control and 100 ppm groups displayed proestrus.

TABLE 39. Terminal Stage of Estrous and Microscopic Kidney Findings[a] F1 Set 1a Males and Females (PND 70)

Observation	Dose group (ppm)			
	Control	100	300	600/800
F1 Set 1a				
MALES				
Degeneration, proximal convoluted tubule, outer strip, outer zone n=	10	10	11	11
● multifocal, very slight	1	1	**6**	**4**
● multifocal, slight	0	0	0	**5**
● focal, very slight	1	2	1	0
FEMALES				
Terminal stage of estrous n=	10	10	10	10
● estrus	3	3	1	3
● diestrus	7	7	7	4
● proestrus	0	0	2	3
Degeneration, proximal convoluted tubule, outer stripe, outer zone	10	10	10	10
● multifocal, very slight	1	0	1	**5**
● multifocal, slight	0	0	0	0
● focal, very slight	1	1	2	1

[a] Data obtained from Text Table 19, page 113 and Appendix Table 87, pages 892-895 in the study report.

C. OFFSPRING: F1 Offspring Set 1b (PND 54-56) – Developmental Neurotoxicity

1. **Body weight (Set 1b F1 Males and Females):** There were no significant differences in body weight or body-weight gain in either sex (Table 40).

TABLE 40. Mean (±SD) F1 Body Weights/Gains of Set 1b (PND 54-56) (g) [a]								
Dose group (ppm)								
Day	**0**	**100**	**300** [B]	**800**	**0**	**100**	**300**	**600**
	F_1 Set 1b – male				**F_1 Set 1b – female**			
21	51.2±3.5	52.4±6.0	51.6±4.0	48.2±4.7 ↓6%	50.7±4.8	48.5±6.3	48.3±4.6	46.0±4.1
28	89.7±4.7	91.7±7.3	89.7±7.7	81.7±7.8* ↓9%	83.8±6.5	81.9±10.4	80.2±7.3	76.4±6.6 ↓9%
56	332.4±23.4	343.1±22.0	337.9±30.1	318.3±24.8	213.2±19.6	214.7±18.8	201.9±22.2	201.7±13.0
21-28	38.5±2.0	39.3±3.7	38.1±5.2	33.4±3.8* ↓13%	33.1±3.2	33.4±4.4	31.9±5.4	30.4±3.4 ↓8%
21-56	281.2±25.1	290.7±17.9	286.3±27.1	270.1±22.2 ↓4%	162.5±18.9	166.2±16.5	153.6±22.6	155.7±10.5 ↓4%

[a] Data obtained from Tables 117-118, pages 399-400 in the study report. n=10; [B] includes one outlier; * α = 0.05; mean ±SD

2. **Functional Observational Battery (FOB):** There was no significant, treatment-related, effect on body weight at any dose level in either sex (Table 41). The only reported difference in FOB observations was in the level of urination in males during the open-field evaluation. There was an increase in the level of urination in all treated male groups compared to the control but there was no dose-response.

   a. Rectal Temperature: There were no treatment-related differences observed in either sex.

   b. Grip Performance: Although there were no statistically significant differences among the groups in either forelimb or hind limb grip performance, both sexes displayed a 10% reduction in hind limb grip performance compared to their respective controls (Table 41). *NOTE: Forelimb grip strength appears to be similar between the sexes, whereas hind limb grip strength is greater in the males than in the females.*

   c. Landing Foot Splay: At the high-dose level in both sexes, males displayed a 17% increase and females displayed a 13% decrease in landing foot splay (Table 41).

Table 41. FOB Parameters – F1 Offspring Set 1b				
Parameter	**0 ppm**	**100 ppm**	**300 ppm**	**600/800 ppm**
Males				
Body weight (g)	332.7±23.3	337.3±21.2	332.2±26.1	319.4±23.8 (↓4%)
Rectal temperature (°C)	38.2±0.5	38.3±0.5	38.4±0.6	37.9±0.7
Hind limb grip (g)	1002.3±154.0	921.8±167.4 (↓8%)	991.1±116.8	904.8±151.2 (↓10%)
Forelimb grip (g)	622.9±140.2	659.2±142.4	662.8±251.6 (↑6%)[B]	608.7±119.4 (↓2%)
Landing foot splay (cm)	9.62±1.71	10.47±1.61	10.49±1.51	11.26±2.33 (↑17%)
Urination	1.1	***2.1***	***1.8***	***1.7***
none	9	2*	4*	5
minimal	1	5	4	3
moderate	0	3	2	2
Females				
Body weight (g)	212.1±20.6	214.8±18.5	200.7±21.1	199.2±14.6 (↓4%)
Rectal temperature (°C)	38.5±0.4	38.6±0.3	38.3±0.6	38.4±0.6
Hind limb grip (g)	714.8±135.1	699.1±121.0	689.8±136.4	649.5±124.1 (↓10%)
Forelimb grip (g)	635.5±145.8	635.9±106.2	584.6±133.7	647.8±79.3
Landing foot splay (cm)	10.31±1.79	9.88±1.83	8.97±1.65	8.94±2.16 (↓13%)
Urination	1.2	1.2	1.3	1.6
none	8	8	8	6

Table 41. FOB Parameters – F1 Offspring Set 1b				
Parameter	**0 ppm**	**100 ppm**	**300 ppm**	**600/800 ppm**
minimal	2	2	1	2
moderate	0	0	1	1

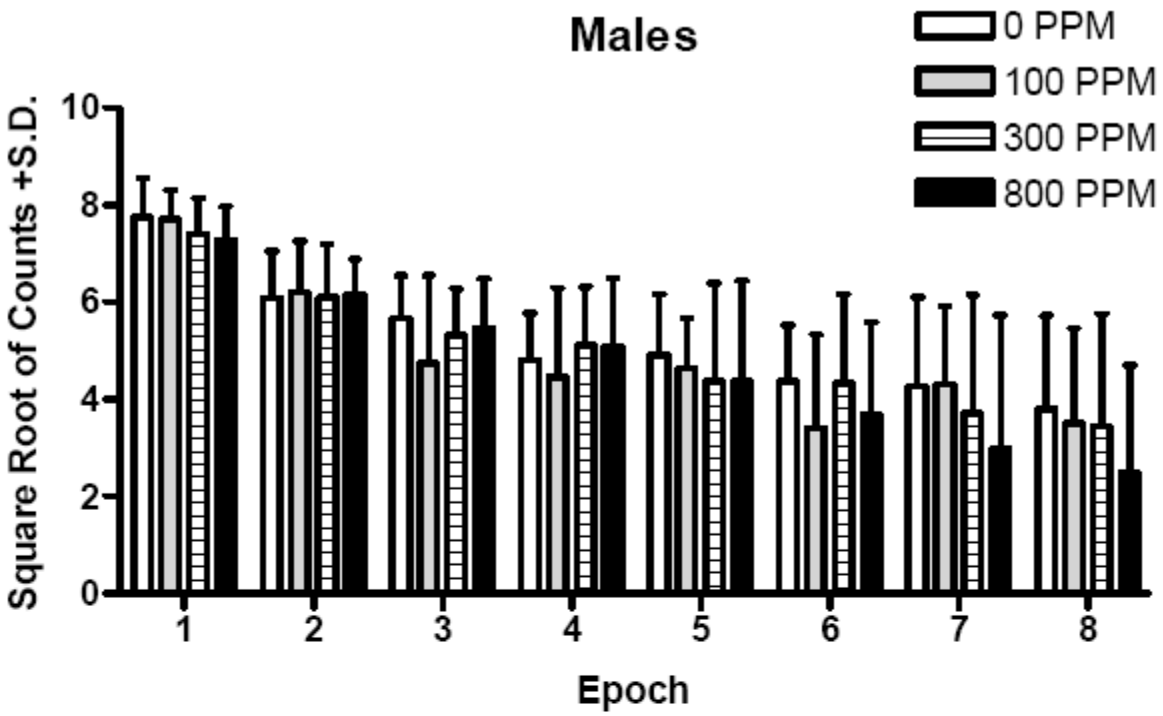
Data from Tables 123-139, pages 405-422 of report; n= 10; α = 0.02; [B] 606.2±187.5 (↓3%) without outlier (1172.3),

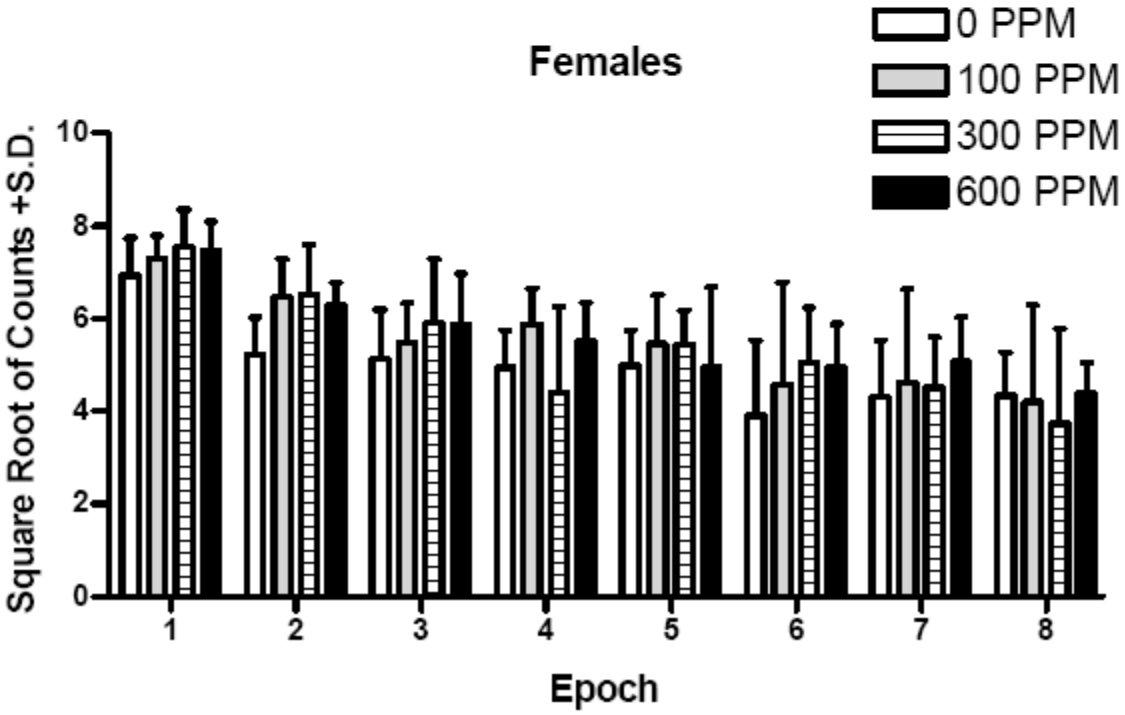
d. Motor Activity: Males: During the first half of the session, all male groups displayed a similar motor activity (MA) level (were within 6%), whereas the 800 ppm males showed a progressive lessening of activity with increased time (Epochs 5 through 8); *i. e.,* the 800 ppm males displayed decreased activity compared to the control (↓11%, ↓16%, ↓30%, and ↓34% in Epochs 5, 6, 7, and 8, respectively, compared to control activity). Total MA counts were reduced (↓10%) in the 800 ppm males compared to the control (Table 42). **Females:** The 600 ppm females displayed a 12% increase in total motor activity compared to the control females. All treated groups displayed slightly greater activity (↑5%-8%) than the control during the 3 initial epochs, although there was no dose-response.

Table 42. Motor Activity Counts per Epoch (square root) and Total MA Counts – Set 1b F1									
Dose (ppm)	**Epoch 1**	**Epoch 2**	**Epoch 3**	**Epoch 4**	**Epoch 5**	**Epoch 6**	**Epoch 7**	**Epoch 8**	**Total**
Males									
0	7.75±0.80	6.09±0.95	5.67±0.88	4.81±0.96	4.91±1.25	4.36±1.16	4.27±1.83	3.80±1.92	41.66±6.99
100	7.71±0.58	6.21±1.05	4.74±1.82	4.46±1.83	4.64±1.03	3.42±1.92	4.30±1.61	3.50±1.96	38.98±5.71
300	7.40±0.74	6.10±1.10	5.32±0.96	5.11±1.20	4.37±2.02	4.33±1.83	3.71±2.44	3.44±2.32	39.76±8.90
800	7.28±0.69	6.16±0.72	5.46±1.03	5.09±1.40	4.36±2.08	3.68±1.91	2.99±2.74	2.49±2.21	37.50±9.80 ↓**10%**
Females									
0	6.92±0.82	5.21±0.81	5.12±1.07	4.94±0.81	4.99±0.76	3.90±1.63	4.30±1.23	4.34±0.93	39.71±3.39
100	7.29±0.50	6.46±0.82	5.47±0.87	5.87±0.78	5.45±1.05	4.57±2.21	4.63±2.01	4.20±2.09	43.93±5.57
300	7.54±0.80	6.52±1.07	5.90±1.38	4.41±1.84	5.44±0.73	5.04±1.19	4.51±1.08	3.73±2.05	43.10±6.52
600	7.45±0.65	6.28±0.50	5.87±1.09	5.52±0.82	4.94±1.73	4.94±0.94	5.07±0.97	4.37±0.67	44.43±4.40 ↑**12%**

Data from Tables 141-144, pages 424-427; mean±s.d.; n=10

FIGURE 26. Motor Activity Epoch Data





3. **Acoustic Startle Response (ASR): Males**: Exposure did not affect the ASR in F1 male (p = 0.5888) Set 1b rats. The interaction of Exposure × Block was statistically significant in males (p = 0.0022), indicating that the habituation of the ASR was significantly affected by exposure. However, subsequent linear contrasts of control and treated groups for the interaction of

Exposure × Block were not statistically significant (Block × 0 ppm vs. 100 ppm, p = 0.1701; Block × 0 ppm vs. 300 ppm, p = 0.0702; Block × 0 ppm vs. 800 ppm, p = 0.0456). The significant Exposure × Block interaction in males was thought to be due to the slightly higher ASR in the first block for the 800 ppm males when compared to controls (Table 43; Figure 26 from study report). This was not interpreted to be a treatment-related effect due to the lack of effect on habituation, lack of statistical significance in the linear contrast when compared to controls, and because the ASR of the 800 ppm males was similar to two recent control data sets from the performing laboratory in male rats of the same strain, age and body weight (Andrus, 2007). **Females**: There were no apparent treatment-related differences in the acoustic startle response in females (Table 43).

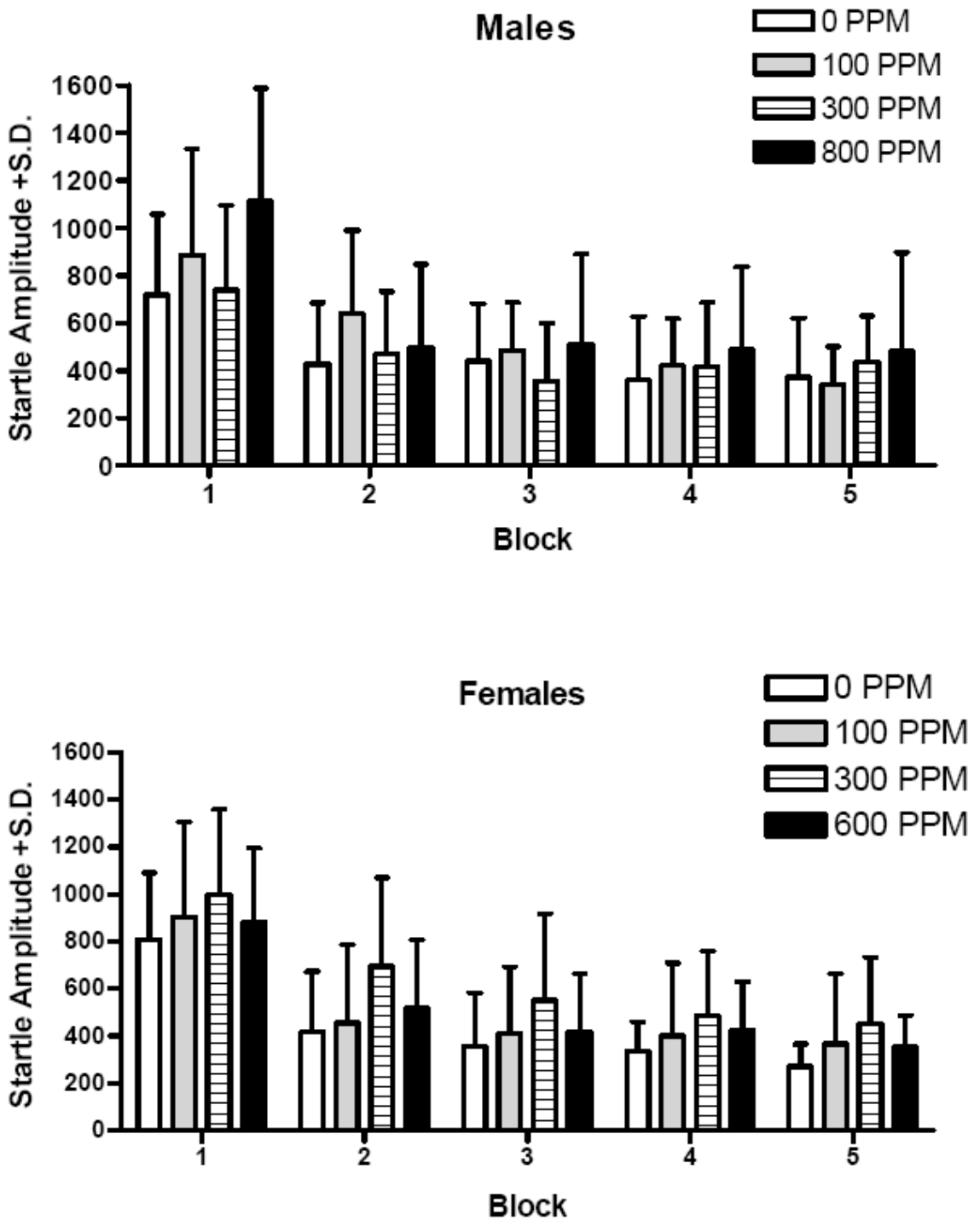
In assessing whether the high-dose males displayed a different response compared to the control, it is noted that one of the 10 control males (the outlier) displayed a value greater than 1000 in Block 1 compared to 3/10, 2/10, and 6/10 males at the 100 ppm, 300 ppm and 600 ppm dose levels, respectively. One 800 ppm male had a value in Block 5 (1327) that was comparable to that in Block 1 (1396). Another 800 ppm male displayed values greater than 1100 in Blocks 1-4.

Table 43. Acoustic Startle Response– F1 Offspring Set 1b

Block #	0 ppm	100 ppm	300 ppm	600/800 ppm
Males				
1	717.6±339.4♪	885.8±446.1	738.6±357.5	1111.6±475.2
2	426.3±257.4♪	639.7±349.0	468.0±265.1	496.1±351.5
3	438.2±245.0♪	485.8±200.5	356.1±243.8♪	509.5±381.5
4	360.0±268.5♪	420.4±197.2	415.0±271.9♪	486.6±349.0
5	371.1±249.5	340.7±159.7	433.8±195.6	480.9±415.6
Females				
1	808.9±280.5	903.1±401.1♪	999.6±358.8	881.9±313.2
2	417.6±256.1	454.9±331.2	692.7±377.4	517.3±288.5♪
3	355.3±228.5	409.6±282.3	551.3±366.3	417.6±245.3♪
4	333.2±125.6	400.7±307.7	484.2±275.4	424.3±204.7
5	269.3±95.5	367.2±293.6	451.6±281.0	353.6±134.7

Data from Tables 146-147, pages 429-430 of the study report; n= 10; ♪ includes statistical outlier

FIGURE 27. ASR Habituation



4. Necropsy/Neuropathology

a. Brain Weight/Gross Measurements: Male and female terminal body weights were comparable among the groups (Table 44). There were no significant differences in perfused absolute brain weights, cerebral lengths and widths, or cerebellar lengths and widths in perfused Set 1b males

or females (PND 60).

Table 44. Brain Weight and Gross Measurements – F1 Offspring Set 1b (perfused)				
	0 ppm	100 ppm	300 ppm	600/800 ppm
Males				
Body weight (g)	320.0±24.0	332.8±25.4	325.2±32.3	310.4±23.7 ↓3%
Brain weight (g)	2.348±0.133	2.395±0.134	2.335±0.109	2.385±0.133
CRL (mm)	16.38±0.33	16.05±0.39	16.17±0.28	16.20±0.28
CRW (mm)	16.58±0.37	16.68±0.39	16.56±0.18	16.62±0.27
CBL (mm)	7.14±0.30	7.21±0.38	7.28±0.321	7.09±0.45
CBW (mm)	12.40±0.49	12.51±0.48	12.52±0.23	12.40±0.31
Females				
Body weight (g)	203.5±17.2	207.7±17.6	195.1±21.1	194.2±11.4 ↓5%
Brain weight (g)	2.228±0.115	2.188±0.157	2.209±0.104	2.152±0.083
CRL (mm)	15.59±0.31	15.30±0.42	15.40±0.27	15.30±0.27
CRW (mm)	16.02±0.35	15.96±0.39	16.19±0.30	15.99±0.29
CBL (mm)	7.60±0.36	7.35±0.26	7.56±0.29	7.43±0.33
CBW (mm)	12.18±0.17	12.21±0.36	12.12±0.27	12.07±0.33

Data from Tables 149-150, pages 432-433 of the study report; n= 10; CRL=cerebrum length; CCRW= cerebrum width; CBL=cerebellum length; CBW= cerebellum width

b. Gross Pathology Observations: There were no gross observations attributed to 2, 4-D in perfused F1 Set 1b male or female rats.

c. Neuropathological Observations: There were no treatment-related microscopic changes in the central or peripheral nervous system in F1 Set 1b perfused male and female rats. This assessment included an evaluation of the cerebellum and corpus collosum for signs of myelin alterations using Luxol Fast Blue. There were no treatment-related changes in myelin. Histopathological observations were interpreted to be spontaneous occurrences common to this strain/age of rat and unrelated to 2, 4-D. Neuropathology was not assessed in the 100 and 300 ppm animals, because of the lack of treatment-related effects at 600 ppm and 800 ppm.

d. Brain Morphometrics (Perfused F1 Set 1b rats): There were no treatment-related changes in microscopic measurements of structures in the cerebral cortex, thalamus, or hippocampus. Due to slight damage of some sections in the cerebellum during dissection, an accurate measurement of one parameter (cerebellar width) was not possible in all samples. However, sample sizes of undamaged cerebellum were considered adequate for evaluation ($n > 6$). There were no significant differences in microscopic cerebellar width measurements and no indications of significant morphometric alterations based on the two additional microscopic measurements in the cerebellum with larger sample sizes. Furthermore, gross measurements of cerebellar length and width did not indicate any significant changes. It was determined that there were no significant treatment-related effects on brain morphometry measurements in F1 Set 1b 800 ppm males and 600 ppm females compared with controls, and morphometric analysis of brain structures was not conducted in the 100 and 300 ppm rats.

C. OFFSPRING: F1 Unselected Offspring (PND 22 Weanlings)

1. Survival: Non-perfused F1 PND 22 Weanlings: There were no effects on the survival of unselected F1 male or female weanlings used for systemic toxicity (non-perfused weanlings). Weanlings designated as unselected F1 weanlings survived to scheduled necropsy.

2. **Organ and Organ/Body Weights: F1 PND 22 Weanlings**: **Males:** Terminal body weights were decreased in all dose groups (9%-10%), although the decrease at the 300 ppm dose level was not statistically significant (Table 45). Decreased absolute (↓37%*) and relative (↓29%) adrenal weights were observed at 600 ppm. Testes weight (absolute) was significantly decreased at all dose levels (14%*-15%*), and relative testes weight was slightly (↓5%) lower at all dose level compared to the control. Absolute spleen weights were significantly decreased (↓18%-26%) at all dose levels, although there was no dose-response. The organ weight effects in both the 100 ppm and 300 ppm males were considered secondary to lower body weights and not treatment related. Additionally, the body weight decrements related to systemic toxicity at 800 ppm contributed to the magnitude of the organ weight decreases in relative liver weights and absolute adrenal, kidney, liver, spleen and testes weights observed at 800 ppm. There were no effects on pituitary, thyroid or brain weights in male weanlings at any dose level. NOTE: *The prostate, seminal vesicles, epididymides, and thymus were not weighed in this group.* **Females:** There were no significant effects reported on terminal body weights or absolute or relative organ weights (adrenal glands, kidneys, liver, brain, spleen, pituitary, and thyroid) at any dose level. It is to be noted that decreased (↓31%) adrenal weights (absolute and relative) were observed at 600 ppm, which is similar in magnitude to the response noted in the males at 800 ppm. NOTE: *The ovaries, uterus, and thymus were not weighed in this group*.

TABLE 45. Organ Weights[a] F1 PND 22 Weanlings (Unselected – General Toxicity)

Organ	Dose group (ppm)			
	Control	100	300	600/800
F1 PND 22 MALES				
Terminal Body Weight (g) N=	57.9±3.8 **10**	52.2±3.8*↓10% **10**	52.8±3.9 ↓9% **10**	51.9±7.4*↓10% **10**
Kidneys ●absolute ●relative	 0.695±0.085 1.200±0.114	 0.582±0.052*↓**16%** 1.117±0.066	 0.614±0.078 ↓**12%** 1.159±0.081	 0.589±0.088*↓**15%** 1.137±0.088
Liver ●absolute ●relative	 2.451±0.322 4.218±0.324	 2.046±0.266* ↓**17%** 3.910±0.282	 2.129±0.231 ↓**13%** 4.035±0.339	 1.998±0.389*↓**18%** 3.832±0.273*↓9%
Thyroid ●absolute ●relative	 0.0063±0.0014 0.0109±0.0022	 0.0054±0.0012 ↓**14%** 0.0104±0.0019	 0.0051±0.0009 ↓**19%** 0.0096±0.0013	 0.0054±0.0011 ↓**14%** 0.0105±0.0015
Testes ●absolute ●relative	 0.278±0.044 0.479±0.067	 0.237±0.024* ↓**15%** 0.454±0.039 ↓5%	 0.239±0.027* ↓**14%** 0.453±0.035 ↓5%	 0.236±0.031*↓**15%** 0.457±0.041 ↓5%
Brain ●absolute ●relative	 1.549±0.091 2.681±0.186	 1.511±0.053 ↓2% 2.905±0.176	 1.492±0.074 ↓4% 2.840±0.237	 1.486±0.070 ↓4% 2.912±0.386
Pituitary ●absolute ●relative	 0.0030±0.0008 0.0052±0.0013	 0.0027±0.0006 ↓**10%** 0.0051±0.0010	 0.0027±0.0009 ↓**10%** 0.0051±0.0017	 0.0027±0.0008 ↓**10%** 0.0052±0.0016
Adrenal glands ●absolute ●relative	 0.0261±0.0080 0.0449±0.0124	 0.0220±0.0066 ↓16% 0.0422±0.0117	 0.0257±0.0490 0.0490±0.0192	 0.0165±0.0037*↓**37%** 0.0318±0.0054↓**29%**
Spleen ●absolute ●relative	 0.282±0.047 0.485±0.060	 0.209±0.045* ↓**26%** 0.398±0.071* ↓**18%**	 0.232±0.028* ↓**18%** 0.440±0.040 ↓**9%**	 0.222±0.050* ↓**21%** 0.424±0.053 ↓**13%**
F1 PND 22 Females				
Terminal Body Weight (g) N=	52.7±6.1 10	50.4±7.9 10	50.5±4.4 10	51.2±5.9 10
Kidneys ●absolute ●relative	 0.629±0.097 1.191±0.081	 0.612±0.099 1.219±0.093	 0.607±0.048 1.214±0.042	 0.599±0.087 ↓5% 1.169±0.070

TABLE 45. Organ Weights[a] F1 PND 22 Weanlings (Unselected – General Toxicity)				
Organ	**Dose group (ppm)**			
	Control	**100**	**300**	**600/800**
Liver				
●absolute	2.138±0.335	2.018±0.419	2.064±0.265	2.059±0.362
●relative	4.046±0.229	3.985±0.331	4.077±0.249	4.002±0.328
Thyroid				
●absolute	0.0059±0.0011	0.0055±0.0015 ↓7%	0.0051±0.0006 ↓14%	0.0055±0.0008 ↓7%
●relative	0.0112±0.0015	0.0109±0.0023	0.0100±0.0014	0.0108±0.0015
Brain				
●absolute	1.491±0.064	1.439±0.065	1.458±0.064	1.438±0.057
●relative	2.862±0.316	2.918±0.439	2.903±0.234	2.842±0.309
Pituitary				
●absolute	0.0030±0.0007	0.0026±0.0004↓14%	0.0025±0.0003 ↓17%	0.0027±0.0003 ↓10%
●relative	0.0057±0.0009	0.0052±0.0009	0.0050±0.0005	0.0052±0.0003
Adrenal				
●absolute	0.0264±0.0081	0.0247±0.0087	0.0251±0.0086	0.0181±0.0046 ↓31%
●relative	0.0511±0.0178	0.0493±0.0165	0.0502±0.0191	0.0353±0.0066 ↓31%
Spleen				
●absolute	0.253±0.034	0.245±0.065	0.213±0.044 ↓16%	0.230±0.050 ↓9%
●relative	0.481±0.038	0.484±0.100	0.421±0.072 ↓12%	0.446±0.063 ↓7%

[a] Data obtained from Tables 79-80, pages 330-333 in the study report. * α = 0.05; mean ±SD

3. **Gross Pathology Observations (F1 PND 22 Weanlings)**: There were no gross observations attributed to 2, 4-D exposure in the unselected F1 weanlings (both sexes).

4. **Histopathological Observations (F1 PND 22 Weanlings):** There were no treatment-related histopathological observations in the weanling rats at 600 ppm/800 ppm (only group examined).

5. **Survival (Perfused F1 PND 22 Weanlings):** There was no treatment-related effect on the survival of unselected weanlings used for perfusions. All unselected F1 weanlings survived to scheduled necropsy.

6. **Brain Weight and Gross Brain Measurements (Perfused F1 PND 22 Weanlings)**: **Males:** Terminal body weights were decreased slightly at the 300 ppm (↓8%) and 800 ppm (↓6%) dose levels, but there was no dose-response. There were no significant differences in perfused absolute brain weights, cerebral lengths and widths, or cerebellar lengths and widths in perfused F1 male weanlings. **Females:** Terminal body weights were not significantly altered in the perfused F1 female weanlings. According to the report, gross brain measures (CRL, CRW, CBL, CBW) in the 100 ppm group were statistically different from controls (multivariate analysis across measures followed by linear contrasts), but because this finding did not follow a dose-response relationship, the result was considered spurious and unrelated to 2, 4-D exposure (Table 46). NOTE: It is not evident to this reviewer how, for example, 15.83±0.32 (control CRW) is statistically different from 15.82±0.34 (100 ppm CRW). The CRW for the 600 ppm females is 15.66±0.43 (↓1%).

Table 46. Brain Weight and Gross Measurements – F1 Weanlings PND 22 (perfused)				
	0 ppm	**100 ppm**	**300 ppm**	**600/800 ppm**
Males				
Body weight (g)	57.1±3.9	56.4±5.7	52.5±4.8 ↓8%	53.7±6.1 ↓6%
Brain weight (g)	1.838±0.092	1.846±0.063	1.829±0.108	1.803±0.085 ↓2%
CRL (mm)	14.84±0.35	14.83±0.28	14.73±0.34	14.55±0.27
CRW (mm)	16.01±0.31	16.08±0.30	16.00±0.37	15.91±0.34
CBL (mm)	6.44±0.27	6.06±0.44 ↓6%	6.11±0.33	5.98±0.26 ↓7%
CBW (mm)	11.92±0.29	11.87±0.32	11.59±0.26	11.75±0.22
Females				
Body weight (g)	54.1±5.4	53.1±4.5	49.9±3.8 ↓8%	50.0±4.3 ↓8%
Brain weight (g)	1.822±0.127	1.740±0.071 ↓5%	1.774±0.075	1.744±0.101
CRL (mm)	14.74±0.41	14.37±0.14 ↓3%	14.40±0.35	14.39±0.29
CRW (mm)	15.83±0.32	15.82±0.34	15.89±0.29	15.66±0.43 ↓1%
CBL (mm)	7.02±0.38	6.82±0.42 ↓3%	6.99±0.23	6.84±0.25
CBW (mm)	11.72±0.30	11.62±0.26 ↓1%	11.67±0.24	11.44±0.37 ↓2%

Data from Tables 83-84, pages 342-343 of the study report; n= 12; CRL=cerebrum length; CCRW= cerebrum width; CBL=cerebellum length; CBW= cerebellum width; linear contrast for multivariate analysis of female 100 ppm group CRL, CRW, CBL, CBW statistically different from control at $\alpha = 0.02$

7. **Gross Pathology Observations: Perfused F1 PND 22 Weanlings:** There were no gross observations attributed to 2, 4-D exposure in either male or female perfused F1 weanlings.

8. **Neuropathological Observations: Perfused F1 PND 22 Weanlings:** There were no neuropathological observations attributed to treatment with 2, 4-D in the perfused F1 PND 22 weanlings. This assessment included an evaluation of myelin using Luxol Fast Blue. The 100 ppm and 300 ppm groups were not examined due to the lack of treatment-related changes in myelin in the 600 ppm/800 ppm PND 22 weanlings.

D. F1 OFFSPRING: Set 2a (PND 67-73) – Developmental Immunotoxicity: Primary Immune Response to Sheep Red Blood Cells (SRBCs)

1. **Survival and clinical signs**: There were no effects on survival. All Set 2a F1 rats survived to scheduled necropsy.

2. **Body Weights/Body Weight Gains: Set 2a F1**: **Males**: A slight decrease in body weight was observed initially in males at the 300 ppm (7%) and 800 ppm (6%) on PND 21, and the 800 ppm males displayed a statistically significant decrease (10%) on PND 28. Body-weight gain during PND 21-28 was decreased by 15% at 800 ppm but was comparable to control thereafter (Table 47). **Females**: Body weights in the 600 ppm females were decreased by 8% on PND 21 and 9% on PND 28. Consistent with these body weight decrements, body weight gains were decreased by 11% in the 600 ppm females from PND 21-28, but were not markedly different from control values thereafter (PND 21-70). There were no exposure-related effects on body weights/body weight gains at 100 ppm and 300 ppm.

TABLE 47. Mean (±SD) F1 Body Weights/Gains of Set 2a (PND 70) (g) [a] Immunotoxicity SRBC								
Dose group (ppm)								
Day	**0**	**100**	**300**	**800**	**0**	**100**	**300**	**600**
	F_1 Set 2a – male				**F_1 Set 2a – female**			
21	52.7±3.9	51.9±5.3	47.8±4.2	49.6±6.2 ↓6%	51.4±4.6	48.7±4.9	50.1±6.3	47.3±3.8 ↓8%
28	90.8±3.9	90.2±6.8	83.1±8.1	81.9±10.4* ↓10%	85.8±5.8	81.5±5.8	84.4±8.1	78.0±6.7 ↓9%
70	410.3±33.2	415.1±25.0	394.0±38.5	398.8±17.2 ↓3%	256.6±23.4	257.7±23.6	254.7±38.8	242.5±13.6 ↓5%
21-28	38.1±2.5	38.4±3.6	35.3±5.5	32.4±8.6 ↓15%	34.4±4.3	32.8±3.0	34.3±3.1	30.8±3.5 ↓10%
21-70	357.6±31.9	363.3±22.4	346.2±37.0	349.3±16.7 ↓2%	205.2±21.3	209.0±24.4	204.6±33.7	195.3±16.1 ↓5

[a] Data obtained from Tables 157-158, pages 447-450 in the study report' n=10

3. **Food consumption (F1 Set 2a Males and Females)**: There were no significant differences in Set 2a male (PND 28-70, ↓2%-5%) and female (PND 28-35, ↓9%) food consumption values at any interval examined, which is consistent with the minimal effects on body weights/body weight gains.

4. **Organ and Organ/Body Weights Summary (F1 Set 2a Males and Females):** Set 2a rats were euthanized on PND 71-73 to minimize inter-assay variability for the SRBC assay. Terminal body weights were comparable among the groups (both sexes). **Males:** Both absolute and relative thymus weights were 17% lower in the 100 ppm males (only relative weight was statistically significant), but there was no dose response relationship and the finding was considered spurious. There were no significant, treatment-related effects on absolute or relative brain, spleen or thymus weights compared to the controls. The positive control displayed a significant loss in absolute (↓44%) and relative (↓41%) spleen weights and absolute (↓73%) and relative (↓72%) thymus weights, consistent with the expected response of a positive control. **Females**: Body weights/gains were comparable among the groups, and there was no effect on terminal body weights at any dose level. There were no statistically significant effects on absolute or relative brain, spleen, or thymus weights, although the thymus weight was decreased at 600 ppm (absolute ↓13% and relative ↓10%), and spleen weights were decreased at 300 ppm (↓13%) and 600 ppm (↓14%/10%). The positive control (cyclophosphamide) showed a significantly decreased terminal body weight (13%), a significant decrease in absolute (47%) and relative (39%) spleen weights, and a significant decrease in absolute (72%) and relative (68%) thymus weights (Table 48).

TABLE 48. Mean (±SD) BodyWeight and Organ Weight – Set 2a F1 Offspring[a]					
Weight (grams)	**Dose group**				
	Control	**+Control**	**100 ppm**	**300 ppm**	**800/600 ppm**
F1 Males – PND 67-73					
Final body weight	424.3±36.1	406.3±30.0 ↓4%	431.1±24.4	408.5±34.6 ↓4%	418.7±17.1
Brain					
●absolute	2.078±0.095	2.052±0.072	2.013±0.063	1.997±0.093 ↓4%	2.024±0.085
●relative	0.492±0.035	0.507±0.036	0.468±0.024	0.491±0.038	0.484±0.029
Spleen					
●absolute	0.889±0.171	0.498±0.021* ↓44%	0.847±0.139	0.827±0.130	0.836±0.138
●relative	0.209±0.029	0.123±0.010 ↓41%	0.196±0.024	0.202±0.026	0.199±0.028
Thymus					
●absolute	0.614±0.109	0.165±0.035* ↓73%	0.512±0.046↓17%	0.625±0.140	0.554±0.060 ↓10%
●relative	0.144±0.019	0.041±0.009* ↓72%	0.119±0.010* ↓17%	0.153±0.031	0.132±0.013 ↓8%

TABLE 48. Mean (±SD) BodyWeight and Organ Weight – Set 2a F1 Offspring[a]					
Weight (grams)	**Dose group**				
	Control	**+Control**	**100 ppm**	**300 ppm**	**800/600 ppm**
F1 Females – PND 67-73					
Final body weight	259.6±21.9	225.8±21.1*↓13%	261.2±22.4	259.0±38.1	252.5±18.2 ↓3%
Brain					
●absolute	1.925±0.070	1.853±0.146	1.910±0.074	1.867±0.086	1.845±0.057 ↓4%
●relative	0.746±0.063	0.828±0.108	0.736±0.067	0.731±0.084	0.734±0.061
Spleen					
●absolute	0.644±0.099	0.343±0.059* ↓47%	0.638±0.094	0.563±0.102↓13%	0.556±0.118 ↓14%
●relative	0.249±0.043	0.151±0.016*↓39%	0.245±0.036	0.217±0.018↓13%	0.219±0.036 ↓12%
Thymus					
●absolute	0.542±0.097	0.153±0.041* ↓72%	0.475±0.092	0.511±0.108	0.473±0.079 ↓13%
●relative	0.208±0.033	0.067±0.014* ↓68%	0.182±0.035	0.197±0.030	0.187±0.025 ↓10%

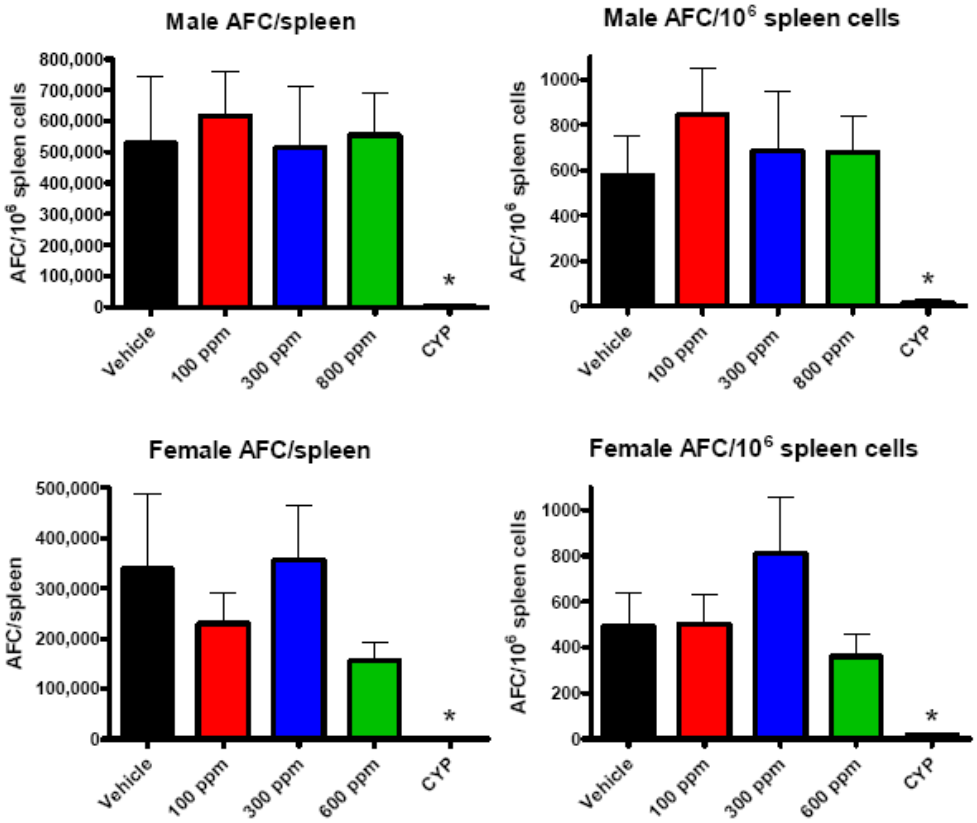
[a] Data obtained from Tables 163-164, pages 455-456 in the study report; n=10; +positive control (n=8); α = 0.05

5. **Primary Immune Response to Sheep Red Blood Cells (SRBCs) Antibody Forming Cell (AFC) assay (F1 Set 2a Rats): Males**: There was no significant difference in response for AFC/spleen and AFC/10^6 splenocytes among the groups of male rats (Table 49). The results for the positive control cyclophosphamide (>95% suppression in both AFC/spleen and AFC/106 splenocytes) showed that the test system was working properly. **Females**: At the 600 ppm dose level, females displayed a non-statistically significant decrease of 54% for the AFC/spleen and 27% for the AFC/10^6 splenocytes. No significant difference in response for AFC/spleen and AFC/106 splenocytes was observed is the 100 ppm and 300 ppm dose females compared to the vehicle control females. This change was not considered to be toxicologically relevant since it occurred at a dose beyond linear toxicokinetics and may have been confounded by the temporal variability introduced by the need to conduct the assay over several days. Analysis of the data by day revealed that days 1 and 2 of the assay yielded a stronger AFC response when compared to days 3 and 4 (dose groups not balanced across days for the assay due to adherence to predefined age restrictions for study conduct). The positive control cyclophosphamide produced >99% suppression in both AFC/spleen and AFC/106 splenocytes.

TABLE 49. SRBC AFC Response – Set 2a F1 Offspring[a] PND 67-73)					
Parameter	**Dose group**				
	Control	**+Control (CYP)**	**100 ppm**	**300 ppm**	**800 ppm**
F1 Males – PND 67-73					
PFC/spleen	528022±645310	3017±7211* ↓>99%	615920±454496	513870±619679	555200±410656
PFC/million	580±524	18±44*↓97%	844±641	684±824	680±473
N=	9	8	10	10	9
F1 Females – PND 67-73					
PFC/spleen	339200±448969	3125±2979* ↓>99%	230330±198225 ↓36%	355950±345431	156020±112661 ↓54%
PFC/million	491±466	19±16* ↓96%	502±398	809±788	360±312 ↓27%
N=	10	8	10	10	10

[a] Data obtained from Tables 165-166, pages 457-458 in the study report; +control; n=8); α = 0.05

FIGURE 32. SRBC AFC Summary – Males and Females



E. F1 OFFSPRING Set 2b (PND 87-93) – Developmental Immunotoxicity: Natural Killer Cell Activity

1. **Survival and clinical signs**: There were no effects on survival. All Set 2b F1 rats survived to scheduled necropsy.

2. **Body Weights/Body Weight Gains**: **Males**: A slight decrease in body weight was observed initially in Set 2b males at 800 ppm (8%) on PND 21. Thereafter, body weights were comparable among the groups (Table 50). Body-weight gain during PND 21-28 was comparable to the control. **Females**: Body weights and body-weight gains were comparable among the groups (Table 50).

TABLE 50. Mean (±SD) F1 Body Weights/Gains of Set 2b (PND 84) (g) [a] Immunotoxicity NK Cell								
Dose group (ppm)								
Day	**0**	**100**	**300**	**800**	**0**	**100**	**300**	**600**
	F_1 Set 2b – male				**F_1 Set 2b – female**			
21	50.0±4.9	52.0±6.4	49.8±4.1	45.8±5.2 ↓8%	48.6±4.8	52.1±6.6	47.5±3.5	46.7±4.0 ↓4%
28	84.0±11.6	90.3±9.5	89.5±6.1	79.8±7.7 ↓5%	81.2±6.1	84.9±9.9	81.0±6.6	77.5±5.7 ↓5%
70	407.3±24.2	427.3±24.3	423.8±27.6	404.3±37.5	238.9±17.1	243.5±16.9	238.1±21.4	244.6±19.2
84	461.0±29.6	488.8±31.6	490.0±31.0	468.9±48.7	262.0±17.5	266.6±14.9	263.1±18.4	269.5±27.3
21-28	34.0±7.6	38.3±3.7	38.8±3.7*	34.0±3.9	32.6±1.6	32.8±5.0	33.5±5.9	30.9±2.8 ↓5%
21-84	411.0±29.3	436.8±28.7	440.2±30.3	423.1±48.1	213.4±16.0	214.5±12.6	215.6±18.9	222.8±28.1

[a] Data obtained from Tables 169-170, pages 461-464 in the study report.

3. **Food consumption:** Food consumption was not adversely affected.

4. **Organ and Organ/Body Weights Summary (F1 Set 2b Males and Females):** Rats were euthanized over a range of days (PND 87-93) to facilitate a balanced study design for each NK cell assay. Terminal body weights were comparable among the groups (both sexes). There were no significant effects on absolute or relative spleen or testes weights (only organs weighed) in the positive control and treated males, and no significant effects on absolute or relative spleen weight (only organ weighed) in the positive control and treated females (Table 51).

TABLE 51. Mean (±SD) Body Weight and Organ Weight – F1 Set 2b Offspring[a]					
Weight (grams)	**Dose group**				
	Control	**+Control**	**100 ppm**	**300 ppm**	**800 ppm**
F1 Males – PND 87-93					
Final body weight	483.0±33.0	449.0±62.1 ↓7%	513.8±33.0	519.4±29.5	495.2±57.7
Spleen					
●absolute	0.749±0.155	0.709±0.100 ↓5%	0.773±0.071	0.804±0.139	0.741±0.135
●relative	0.154±0.025	0.159±0.017	0.151±0.015	0.155±0.024	0.149±0.017
Testes					
●absolute	3.575±0.168	3.416±0.097 ↓4%	3.476±0.387	3.560±0.239	3.267±0.326 ↓9%
●relative	0.743±0.062	0.772±0.096	0.677±0.073	0.686±0.045	0.664±0.073
F1 Females – PND 87-93					
Final body weight	269.3±16.8	253.0±22.1 ↓6%	278.7±16.9	272.1±17.0	281.1±30.1
Spleen					
●absolute	0.495±0.096	0.467±0.036 ↓6%	0.505±0.066	0.517±0.048	0.507±0.071
●relative	0.184±0.032	0.185±0.013	0.182±0.025	0.190±0.019	0.181±0.020

[a] Data obtained from Tables 175-176, pages 469-470 in the study report; n=10; +positive control (n=8)

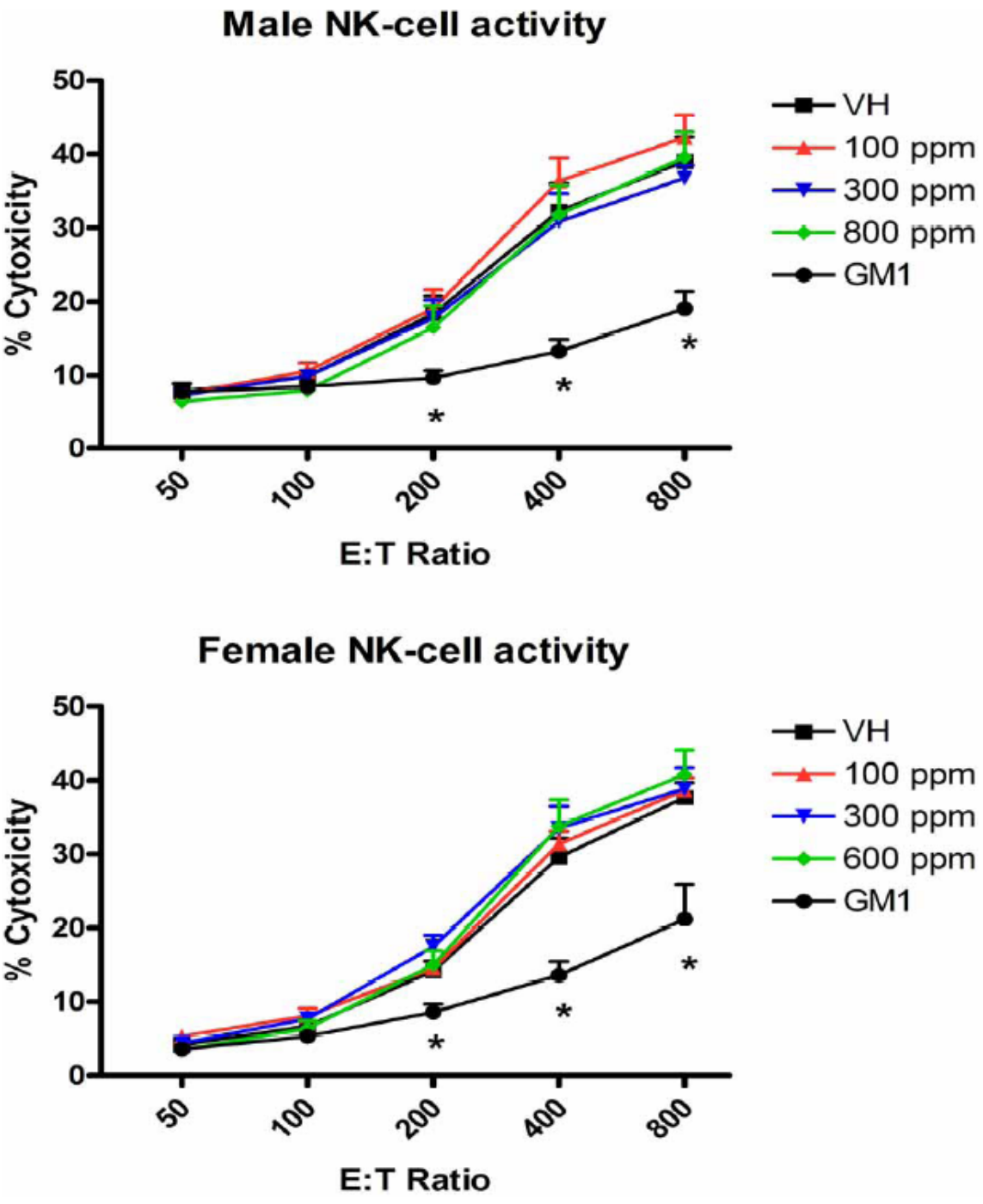
5. **Natural Killer Cell Activity – F1 Set 2b Males and Females:** There was no significant difference in percent target cell cytotoxicity at any dose level compared to the control (both sexes), whereas anti-asialo-GM1 (positive control) produced a significant 40–60% suppression of NK cell activity at the 400:1 and 200:1 effector-to-target ratios in both males and females (Table 52; Figure 33, copied from the study report, page 222), demonstrating the expected response. Based on these results, 2, 4-D did not alter the cytotoxic ability of splenic NK-cells in male or female rats at any dose levels.

TABLE 52. NK Cell Activity – F1 et 2b Offspring[a] (PND 87-93) - percent target cell cytotoxicity

Effector-to-target cell ratios	Dose group				
	Control	+Control (1:5GM1)	100 ppm	300 ppm	800 ppm
F1 Males – PND 87-93					
NK % 50:1 RATIO	7.8±2.7	7.8±3.1	7.6±2.8	7.4±2.2	6.5±1.7 ↓17%
NK % 100:1 RATIO	9.9±2.5	8.5±2.5	10.6±3.6	9.9±2.3	8.0±1.3 ↓19%
NK % 200:1 RATIO	18.5±8.2	9.7±2.8*	19.2±8.5	17.8±7.1	16.5±9.9 ↓11%
NK % 400:1 RATIO	32.4±12.7	13.3±4.6*	36.5±10.3	30.9±7.5	31.8±13.4
NK % 800:1 RATIO	39.0±11.3	19.1±6.6*	42.3±9.7	36.8±5.7	39.6±11.6
F1 Females – PND 87-93					
NK % 50:1 RATIO	4.3±3.2	3.7±0.7	5.3±2.5	4.4±2.2	3.5±2.1 ↓19%
NK % 100:1 RATIO	6.7±2.9	5.3±1.2	8.1±3.3	7.7±2.7	6.5±3.1
NK % 200:1 RATIO	14.3±4.5	8.7±3.2*	14.5±2.0	17.5±5.0	15.1±6.4
NK % 400:1 RATIO	29.7±8.7	13.6±5.3*	31.4±5.8	33.5±10.0	33.8±12.0
NK % 800:1 RATIO	37.7±6.7	21.2±13.4*	38.7±5.4	39.0±9.6	40.8±10.8

[a] Data obtained from Tables 177-178, pages 471-472 in the study report; n=10; +control (anti asialo GM1; n=8); α = 0.05

FIGURE 33. NK Cell Activity Summary – Males and Females



F. F1 OFFSPRING: Set 3 (PND 139) - Reproductive Toxicity

1. **Survival and clinical signs**: The were no compound-related effects on survival or clinical signs..

2. **Body weight (F1 Set 3 Males and Females): Males:** After weaning, body weights in the 800 ppm males were slightly decreased (≈↓7%) initially but were similar to control body weights at study termination (PND 139). Consistent with these reductions in body weight, body weight gains were decreased (↓≈6%-12%) from PND 21-49 but were similar to control values overall. Body weight/body weight gains were comparable to control values at 300 ppm, but significant increases in body weight (↑5%-7%) and body-weight gain (↑6%-7%) were observed at 100 ppm during PND 84-137. **Females:** Initially, a slight decrease in body weight (PND 21 ↓7%; PND 28 ↓8%) was observed at 600 ppm compared to the control, and body weight gains were decreased by ↓10% (PND 21-28). Comparable body weights/gains were observed at 600 ppm compared to control during the rest of the treatment period. There were no significant treatment-related effects on body weights/body weight gains at 100 ppm and 300 ppm (Table 53).

TABLE 53. Mean (±SD) Body weight – F1 Offspring Set 3 Post-Weaning [a]

Observations/study week	Dose group			
	Control	100 ppm	300 ppm	800/600 ppm
F1 males – Set 3				
Mean body weight (g)				
Day 21	52.0±4.9	51.7±5.3	50.3±6.1	49.9±5.4 ↓4%
Day 28	89.6±9.8	90.0±7.4	88.0±8.6	83.1±10.8*↓7%
Day 35	150.7±12.3	150.5±10.7	147.4±12.3	140.0±15.1*↓7%
Day 137	606.7±50.4	644.0±50.7*	614.4±64.3	605.9±57.6
Mean weight gain (g)				
Days 21-28	37.6±6.4	38.3±3.2	37.8±4.6	33.2±8.1*↓12%
Days 21-137	554.7±48.6	592.2±49.4*	563.8±62.8	556.0±59.0
F1 females – Set 3				
Mean body weight (g)				
Day 21	49.7±5.2	49.4±4.9	48.7±5.7	46.0±4.2*↓7%
Day 28	83.4±6.2	82.3±5.4	81.3±7.7	76.7±6.6*↓8%
Day 35	128.0±9.4	127.1±6.5	126.4±10.1	121.6±9.9 ↓5%
Day 137	316.6±29.5	316.0±23.6	322.3±29.2	316.9±35.4
Mean weight gain (g)				
Days 21-28	33.7±3.5	32.8±2.3	32.7±3.3	30.6±3.8*↓10%
Days 21-137	266.9±28.6	266.6±22.5	273.8±28.9	270.8±33.6

[a] Data obtained from Tables 181-182, pages 476-481in the study report.
* α <0.05. ** α <0.01. n = 27 (males)/39 (females; main +satellite)

3. **Feed Consumption – F1 Set 3 Males and Females**: Decreased food consumption was observed in the 800 ppm males (6%-8%) over the interval from PND 28-49, which is consistent with the decreased body weight observed in this group. There was no significant difference in food consumption among the female groups.

4. **Estrous cycle length: F1 Set 3 Females.** No significant difference was observed in mean estrous cycle length in females at any dose level compared to the control (Table 54). There was no indication of persistent estrus; i.e., greater than 2 consecutive days in estrus, and no apparent difference in the percentage of time spent in estrus or diestrus (see Figure 13 in Appendix B).

TABLE 54. Estrous cycle data[a] (F1 Set 3 Females)				
Mean days per cycle	**Dose group**			
	Control	**100 ppm**	**300 ppm**	**600 ppm**
4.0	8	15	10	15
4.2	4	3	1	2
4.3	1	2	3	-
4.4	1	1	1	1
4.5	2	1	-	-
4.6	2	2	3	-
4.8	5	3	2	4
5.0	4	-	-	2
5.2	-	-	1	-
5.3	-	-	1	-
5.7	-	-	1#	-
mean±sd	4.4±0.4	4.2±0.3	4.4±0.5 (23)	4.3±0.4 (24)

[a] Data obtained from Table 187, page 488 and Appendix Table 144, pages 1136-1139 in study report. n=27, except (); # statistical outlier

5. **Ovarian follicle counts – F1 Offspring Set 3 females:** There were no significant, treatment-related effects on the numbers of small follicles, growing follicles, or total follicles (small + growing) at 600 ppm. Due to the lack of effect at 600 ppm, ovarian follicle counts were not conducted for the 100 ppm and 300 ppm samples (Table 55).

Table 55. Ovarian Follicle Counts – F1 Set 3 Females			
Dose (ppm)	**Small**	**Growing**	**Total**
0	66±27	25±10	92±26
600	66±26	28±7	94±28

Data from Table 194, page 499 of the study report; n=15

6. **Sperm Analyses – F1 Offspring Set 3:** There were no significant, treatment-related effects on sperm motility or progressive motility (Table 56). With respect to epididymal sperm and testicular spermatid counts, there were no significant, treatment-related differences between 0 and 800 ppm males. Due to the lack of effect at 800 ppm, sperm/spermatid counts were not conducted for the 100 and 300 ppm groups. Similarly, there were no significant, treatment-related, changes in sperm morphology in the 800 ppm group compared with the control group. While the proportion of abnormal sperm was slightly higher in the 800 ppm males (0.025 vs. 0.016 in controls), this result was due to a single outlier value in the 800 ppm group (0.24). Removal of this outlier value resulted in an equivalent value for mean proportion of abnormal sperm between the 800 ppm and control groups (abnormal sperm = 0.016 in both groups). Sperm morphology was not examined in samples from the 100 ppm and 300 ppm groups due to the lack of effect at 800 ppm.

TABLE 56. Sperm data[a] (F1 Set 3 males)				
Dose group				
Parameter	**Control**	**100 ppm**	**300 ppm**	**800 ppm**
Motile (%)	96.8±4.6	97.4±2.6	97.4±2.6 [A]	97.4±2.3 [B]
Progressively Motile (%)	88.0±7.5	86.5±4.8	86.6±4.9 [A]	87.4±4.0 [B]
Testicular spermatid counts				
Total Sperm (10^{06})	344.2±106.3	-	-	322.5±72.4 [B]
Conc/g (10^{06})	184.7±49.5	-	-	176.2±44.8 [B]
Epididymal sperm counts				
Total Sperm (10^{06})	278.5±92.9	-	-	292.7±87.1 [B]

TABLE 56. Sperm data[a] (F1 Set 3 males)				
	Dose group			
Parameter	Control	100 ppm	300 ppm	800 ppm
Conc/g (10^{06})	833.7±263.9	-	-	895.6±228.1 [B]
Proportion of abnormal sperm				
Abnormal sperm/total	0.016±0.012	-	-	0.025±0.047 [B]

[a] Data obtained from Tables 190-193, pages 495-498 in the study report; n=27, except [A] (n=23); [B] (24)

7. **Organ weights (F1 Set 3): Males** – There was no adverse effect on terminal body weights in the males. There were no significant, treatment-related effects on absolute or relative weights of the adrenal glands, brain, kidneys, liver, prostate, spleen, testes, thymus, epididymides, seminal vesicle or thyroid glands. Absolute (↓9%) and relative (↓8%) pituitary gland weights were significantly lower at 800 ppm (Table 57). The magnitude of the differences from pituitary weights in control rats are minimal (800 ppm: absolute 0.0127 g and relative 0.0022 g/100 g body weight; control: absolute 0.0139 g and relative 0.0024 g/100 g body weight), and both the absolute and relative pituitary weights were within the historical control range for the testing facility. Additionally, there was no associated histopathology in the pituitary glands.

Females - There was no adverse effect on terminal body weights in the females. Absolute and relative kidney weights were increased in 600 ppm females (↑8%), although the relative difference did not attain statistical significance. This finding was consistent with kidney weight increases in other groups, had a histopathological correlate, and was considered treatment related, although not adverse. Increased uterine weight (absolute ↑10% and relative ↑11%) was observed in the 600 ppm females, which is consistent with the increase observed in the P1 females (LD 22) and F1 Set 1a females (PND 70). Pituitary weights were decreased (absolute ↓9% and relative ↓10%) in the 600 ppm Set 3 females, but statistical significance was not attained. Additionally, thymus weight (absolute ↓14% and relative ↓13%) was decreased in the 600 ppm Set 3 females, but statistical significance was not attained. There were no significant, treatment-related, effects on absolute or relative weights of the adrenal glands, liver, brain, ovaries, spleen, or thyroid glands (Table 57).

TABLE 57. Organ Weights[a] F1 Set 3 Offspring				
Organ	Dose group (ppm)			
	Control	100	300	600/800
F1 Set 3 Males (PND 139)				
Terminal Body Weight (g)	578.7±48.2	616.1±49.3*	585.3±64.6	577.4±55.8
N=	27	27	23	24
Kidneys				
●absolute	4.005±0.415	4.171±0.404	4.167±0.401	4.094±0.392
●relative	0.693±0.060	0.678±0.059	0.717±0.081	0.712±0.064
Liver				
●absolute	16.176±2.144	17.296±2.425	16.043±2.395	15.491±1.832 ↓4%
●relative	2.788±0.190	2.801±0.256	2.734±0.202	2.683±0.197
Thyroid				
●absolute	0.0269±0.0040	0.0279±0.0047	0.0265±0.0048	0.0259±0.0049↓4%
●relative	0.0046±0.0006	0.0045±0.0007	0.0045±0.0007	0.0045±0.0008
Testes				
●absolute	3.743±0.305	3.801±0.387	3.684±0.287	3.725±0.527
●relative	0.651±0.075	0.620±0.071	0.636±0.079	0.645±0.057
Prostate				
●absolute	1.041±0.199	1.075±0.241	1.081±0.215	1.070±0.181
●relative	0.181±0.038	0.174±0.035	0.186±0.036	0.187±0.037
Seminal vesicles				
●absolute	1.807±0.287	1.741±0.247	1.760±0.376	1.761±0.286

TABLE 57. Organ Weights[a] F1 Set 3 Offspring

Organ	Dose group (ppm) Control	100	300	600/800
●relative	0.313±0.047	0.284±0.045	0.304±0.072	0.308±0.060
Epididymides				
●absolute	1.399±0.125	1.386±0.113	1.375±0.091	1.388±0.088
●relative	0.244±0.031	0.226±0.023	0.237±0.029	0.243±0.028
Brain				
●absolute	2.158±0.091	2.146±0.069	2.139±0.085	2.123±0.093
●relative	0.375±0.030	0.350±0.030*	0.369±0.039	0.370±0.033
Pituitary				
●absolute	0.0139±0.0018	0.0143±0.0015	0.0137±0.0012	0.0127±0.0014* **↓9%**
●relative	0.0024±0.0002	0.0023±0.0003	0.0024±0.0003	0.0022±0.0002* **↓8%**
Thymus				
●absolute	0.322±0.058	0.323±0.084	0.331±0.125	0.305±0.060 **↓5%**
●relative	0.056±0.010	0.053±0.012	0.056±0.020	0.053±0.010
Adrenal				
●absolute	0.060±0.010	0.067±0.009	0.066±0.010	0.061±0.011
●relative	0.010±0.002	0.011±0.001	0.011±0.002	0.011±0.002
F1 Set 3 Females (PND 139)				
Terminal Body Weight (g)	299.5±27.3	299.0±23.3	304.3±27.7	300.0±33.6
N=	27	27	23	24
Kidneys				
●absolute	2.080±0.212	2.146±0.173	2.199±0.192	2.241±0.218* **↑8%**
●relative	0.698±0.084	0.720±0.061	0.726±0.067	0.751±0.069 **↑8%**
Liver				
●absolute	8.063±1.023	7.999±0.760	8.033±0.862	8.047±0.773
●relative	2.693±0.252	2.676±0.157	2.641±0.183	2.698±0.237
Thyroid				
●absolute	0.0177±0.0030	0.0191±0.0029	0.0188±0.0035	0.0187±0.0029
●relative	0.0059±0.0009	0.0064±0.0010	0.0062±0.0010	0.0063±0.0011
Ovaries				
●absolute	0.089±0.023	0.096±0.020	0.094±0.015	0.091±0.014
●relative	0.030±0.008	0.032±0.007	0.031±0.005	0.031±0.005
Uterus				
●absolute	0.679±0.245	0.734±0.280	0.750±0.309 ↑10%	0.750±0.244 **↑10%**
●relative	0.228±0.082	0.245±0.090	0.250±0.109 ↑10%	0.252±0.084 **↑11%**
Brain				
●absolute	1.971±0.084	1.933±0.064	1.978±0.080	1.925±0.066
●relative	0.662±0.055	0.650±0.047	0.654±0.056	0.649±0.069
Pituitary				
●absolute	0.0152±0.0035	0.0149±0.0021	0.0145±0.0031	0.0139±0.0022 **↓9%**
●relative	0.0051±0.0012	0.0050±0.0007	0.0048±0.0011	0.0046±0.0006 **↓10%**
Thymus				
●absolute	0.280±0.060	0.262±0.062	0.296±0.059	0.242±0.043 **↓14%**
●relative	0.093±0.016	0.088±0.020	0.097±0.018	0.081±0.013 **↓13%**
Adrenal				
●absolute	0.066±0.010	0.070±0.009	0.068±0.011	0.065±0.013
●relative	0.022±0.004	0.024±0.004	0.023±0.004	0.022±0.004

[a] Data obtained from Tables 188-189, pages 489-494 in the study report. * α = 0.05; mean ±SD

8. Gross Pathology - F1 Set 3 Males and Females

There were no treatment-related findings at necropsy.

9. Histopathological Observations (F1 Set 3 Males and Females)

Microscopic examination: Histopathological examinations were conducted on 10 randomly selected control and high-dose rats/sex, with the exception of the testis and epididymis, which were examined in all F1 Set 3 males of the control and high-dose groups (PND 139). Effects were observed on the kidney in male rats at 300 ppm and 800 ppm and in females at 600 ppm

(Table 58). The effect was described as a degenerative lesion involving the proximal convoluted tubules in the outer stripe of the outer zone of the medulla, which was multifocal in distribution, and very slight degree in females and slight degree in males. No histopathological changes were observed in the thymus or pituitary in either sex. There were no apparent treatment-related lesions in the reproductive organs/tissues.

TABLE 58. Microscopic Kidney Findings[a] F1 Set 3 Males and Females

Observation	Dose group (ppm)			
	Control	100	300	600/800
F1 Set 3				
MALES				
Number of kidneys examined	10	10	11	11
Degeneration, proximal convoluted tubule, outer strip, outer zone				
● multifocal, very slight	10	10	**8**	**4**
● multifocal, slight	0	0	**3**	**6**
FEMALES				
Number of kidneys examined	10	10	10	10
Degeneration, proximal convoluted tubule, outer stripe, outer zone				
● multifocal, very slight	0	0	1	**7**
● multifocal, slight	0	0	0	0

[a] Data obtained from Text Table 20, page 130 in the study report.

III. DISCUSSION AND CONCLUSIONS:

INVESTIGATORS' CONCLUSIONS: Systemic toxicity was assessed across life stages. In the parental generation, high-dose females had decreased body weight on LD 7, which coincided with decreased feed consumption (LD 1-7) and decreased high-dose F1 pup body weights. The F1 pup body weights in the high-dose group recovered to control values by ~ PND 42 (females) and 56 (males). The study confirmed that the kidneys were a target organ for 2, 4-D toxicity. P1 males had increased kidney weights and very slight to slight degeneration of the proximal convoluted tubules in the outer zone of the medulla at 800 ppm. Similar exposure-related histopathological renal lesions occurred in the 300 ppm and 800 ppm F1 adult males and 600 ppm F1 adult females. No exposure-related kidney lesions were seen in PND 22 F1 pups. The higher incidence of the kidney histopathology effects in the F1 adults compared to P1 adults was likely related to higher 2, 4-D doses in F1 offspring and was associated with non-linear TK. These kidney lesions were slight to very slight in severity and would not be expected to alter renal function, confirmed by the lack of exposure-related effects on clinical pathology (urea nitrogen and creatinine) or urinalysis (volume and specific gravity).

With respect to reproductive toxicity, 2,4-D had no effects on estrous cyclicity (P1, satellite GD 17 dams, F1 Set 3 offspring) or reproductive indices, including mating, fertility, time to mating, gestation length, pre- and post-implantation loss and corpora lutea number (satellite GD 17 dams). Litter sizes, pup survival, sperm parameters, ovarian follicle counts, and reproductive organ histopathology were unaffected by 2, 4-D. One P1 male in each of the 300 and 800-ppm groups had decreased bilateral testis size, but this incidence rate was within the laboratory historical control data. A similar unilateral finding was noted in one control F1 male. These findings were not reproduced in F1 offspring Set 1a or Set 3 with longer 2, 4-D exposures, which included higher mg/kg/day exposures during critical life stages. There were no exposure-related histopathological changes in reproductive organs. Overall, there was no indication of reproductive toxicity by 2, 4-D in this study. Based on *a priori* established criteria, mating of a second generation was not triggered.

In P1 males, decreased seminal vesicle and prostate weights were seen at > 300 ppm; however, there was no associated histopathology, and absolute and relative control organ weights exceeded the laboratory historical control data. These findings were not reproduced in Set 1a or Set 3 F1 offspring with longer 2, 4-D exposures, which included higher mg/kg/day exposures during critical life stages. Therefore, these organ weight differences were not attributed to 2, 4-D exposure. Decreased testis weights in PND 22 F1 weanlings at all exposure levels, which lacked corresponding histopathology, were attributed to decreased body weights, which were artifactual differences in PND 22 male pup body weights that were introduced during group assignment at weaning. These body weight differences may have been exacerbated by 2, 4-D-related toxicity and/or palatability issues in the 600 ppm group. Available data predicted that this dose group would have exceeded saturation kinetics, despite adjustment of dietary concentrations during lactation, due to the presence of 2, 4-D in milk and in the diet, and the high dietary intakes on a mg/kg basis for this life stage. There were no significant, exposure-related changes in reproductive organ weights in P1 or F1 PND 22 females, or in F1 Set 1a, Set 2 (testis weights only), or Set 3 males and females. There was a slight delay in F1 preputial separation at 800 ppm, which was attributed to high-dose body weight decrements. Other androgen-sensitive endpoints, including anogenital distance (AGD) and nipple retention, which are considered very sensitive endpoints, sperm parameters and testicular and accessory sex gland histopathology, were not altered. There were no effects on estrogen-sensitive endpoints including vaginal opening, estrous cyclicity, uterine weights or histopathology, or quantitative ovarian follicle counts. There was no consistent pattern of effects on thyroid parameters. High-dose satellite GD 17 dams given 600 ppm 2, 4-D had non-significant decreases in T4 and T3 with a corresponding increase in TSH. Thyroid histopathological alterations were seen in three of 12 dams, comprised of smaller thyroid follicles with small vacuoles in the colloid that were suggestive of colloid resorption. There were no adverse pathological alterations (*e.g.*, degeneration); thus, these thyroid changes were considered adaptive. There were no thyroid effects at lower-dose levels in GD 17 dams and no biologically significant effects on thyroid endpoints at the other life stages examined. Thus, 2, 4-D did not alter estrogen or androgen-sensitive endpoints, and altered thyroid endpoints only at an exposure level (600 ppm) that clearly resulted in nonlinear TK.

With respect to developmental neurotoxicity (DNT), there were no exposure-related effects on FOB parameters, motor activity, or acoustic startle response (ASR). There was a significant EXPOSURE x BLOCK interaction for ASR in high-dose males, which was attributed to a slightly higher ASR in the first block. Habituation was not affected at any dose of 2, 4-D. Gross and morphometric brain measurements also were not affected. There was no exposure-related neuropathology in PND 22 or PND 60 animals, including no effects on brain myelin (assessed by special staining). Related neurotoxicity endpoints were examined in P1 adults and other F1 offspring, including brain weights and/or histopathology in non-perfused animals (P1 adults and PND 22, Set 1a on PND 70, and Set 3 on PND 139). Terminal body weights and absolute brain weights were significantly decreased in PND 70 high-dose males; however, this group had a smaller sample size (n=10/sex/dose) than other groups with no brain weight changes (P1 adults and F1 Set 3 on PND 139; both with n>23/sex/dose). There were no exposure-related effects on brain histopathology at any age. Thus, there was no evidence of DNT related to 2, 4-D exposure.

With respect to developmental immunotoxicity, 2, 4-D had no effect on Antibody Forming Cell (AFC) assay responses in males. High-dose females had non-significant decreases in AFC/spleen and AFC/10_6 splenocytes (54% and 27%, respectively). However, this observation appeared to be due to temporal variability over the 4-day span of the evaluations. In addition, this result occurred at 600 ppm, a dose level that demonstrated non-linear TK. The AFC response was not altered at < 300

ppm in females. The F1 Natural Killer Cell activity (NK) assay showed no effects from 2, 4-D exposure. The assay showed linear cytotoxicity with increasing E:T cell ratios (from 5 to 40% cytotoxicity), which were identical across all doses. Overall, 2, 4-D exposure had no biologically significant effect on the development of immune function.

The 2, 4-D F1-extended one generation study has established across life stages a no observed adverse effect level (NOAEL) based on systemic toxicity of 100 ppm in males (5.5 mg/kg/day) and 300 ppm in females (20.6 mg/kg/day in non-pregnant P1 adults). The male NOAEL is ~13,000-fold higher than 2, 4-D exposures reported in human biomonitoring studies. Very slight to slight, renal tubular degeneration was seen in P1 males at 800 ppm and in F1 males exposed >300 ppm 2, 4-D. The increased incidence of renal changes in the F1 adult males compared to the P1 males was likely related to their higher feed consumption and consequent test material intake prior to adulthood. For females, toxicity was limited to the 600 ppm group, where nonlinear TK prevailed, making these results irrelevant for 2, 4-D risk assessment.

B. REVIEWER COMMENTS: Potential Estrogenic Effects: There was no treatment-related effect on developmental landmarks. AGD, which was measured in all pups, nipple retention (all non-culled pups), and age at vaginal opening (all Set 1-3 offspring) were comparable among the groups. There were no adverse effects on estrous cycle length or estrous cycle pattern, including a lack of persistent estrus (assessed in all P1 main study and satellite GD 17 females and all F1 Set3 females). Reproductive indices were comparable among the groups [mating, fertility, time to mating, gestation length, pre-implantation loss, number of corpora lutea (satellite GD 17 dams), sperm parameters, ovarian follicle counts (F1 Set 3 females), and reproductive organ weights (P1, F1 offspring Sets 1a or 3) and histopathology]. There were no signs of dystocia in P1 dams and no adverse effects on post-implantation loss, litter size, and pup survival. In all three age groups in which uterine weights were measured [P1 (LD 22), F1 Set 1a (PND 70), and F1 Set 3 (PND 139)], increased absolute and relative uterine weights (10%-32%) were observed at 600 ppm. There were no significant, treatment-related changes in other reproductive organ weights in P1, PND 22, Set 1a or Set 3 females.

Androgen-Sensitive Endpoints: There were no adverse effects on anogenital distance or nipple retention in any F1 males exposed *in utero*, during lactation and into adulthood. There was no evidence of hypospadias, ectopic testes, or treatment-related testicular, prostate or seminal vesicle histopathology. In P1 and F1 Set 3 (PND 139) males, there were no significant changes in sperm parameters (spermatid/sperm counts, sperm motility and sperm morphology). Decreased accessory sex gland weights were observed in the P1 males, but there were no effects on reproductive or accessory sex gland weights in the F1 generation males when assessed at PND 70 (Set 1a), PND 87-93 (Set 2b) or PND 139 (Set 3). There were no treatment-related effects on reproductive organ histopathology (P1, Set 1a, Set 3, F1 weanlings).

Anti-Androgenic Effects: Decreased male reproductive organ weights (testes and seminal vesicle) were observed in P1 males, although statistical significance was not attained. Flaccid testes were observed in one 300 ppm P1 male and one 800 ppm P1 male. Decreased testes weight was observed at all dose levels (same magnitude) in one subset of weanlings (F1 PND 22) and was associated with decreased body weight. There were no effects on reproductive or accessory sex gland weights in the F1 offspring when assessed at PND 70 (Set 1a), PND 87-93 (Set 2b), or PND 139 (Set 3). Delayed preputial separation (1.6 days) was observed in F1 weanlings, which is within normal variability and was accompanied by a slight reduction in body weight. There was no associated histopathology in P1 male reproductive or accessory sex gland tissues, and reproductive function was not affected. In F1 males, exposed *in utero*, during lactation and into adulthood, there were no effects on anogenital distance or nipple retention, two endpoints that are considered highly sensitive to altered androgen status. There were no treatment-related effects on reproductive organ histopathology (Set 1a, Set 3, F1 weanlings).

Thyroid: GD 17 satellite dams displayed the expected hormone response consistent with a perturbation in thyroid function. These findings correlated with the thyroid alterations observed histologically. There was a comparable increase (↑9%) in thyroid weights at the 100 ppm and 600 ppm dose levels but no change in thyroid weight at 300 ppm. F1 PND 4 pups displayed a slight decrease in T3 (males) and T4 (both sexes; no dose response) and an increase in TSH (females at 600 ppm). Thyroid weight and histopathology data were not collected for this age group. F1 offspring PND 22 displayed decrease T3 (300 ppm and 800 ppm males) and T4 (both sexes 800 ppm/600 ppm) and increased TSH (300 ppm males). Thyroid weight and histopathology data were not collected for this age group. F1 Offspring PND 62-64 displayed increased TSH at 300 ppm and 800 ppm/600 ppm (both sexes), although a dose-response was observed only in the females. Decreased T4 was observed only in the 800 ppm males, and decreased T3 was observed at all dose levels in males but there was no dose-response. F1 Set 1a male offspring (PND 70) displayed decreased absolute thyroid weights (↓11%) at 800 ppm, but terminal body weight was also decreased (↓10%). F1 Set 1a female offspring (PND 70) displayed decreased absolute thyroid weights at 300 ppm (↓9%) and 600 ppm (↓5%). There were no histopathological findings reported in the thyroids. These findings suggest that 2, 4-D exposure may adversely affect thyroid function at doses above the renal saturation clearance; however, the thyroid effects noted below renal saturation are not considered sufficiently robust to be adverse.

Developmental Neurotoxicity (DNT): F1 Set 1b offspring were examined on PND 54-59. There was a lack of evidence of DNT (FOB parameters, motor activity, and acoustic startle response). PND 22 pups and PND 60 F1 adult rats were perfused for central and peripheral nervous system neuropathology, and brain weights, gross brain measurements (PND 22, PND 60) and morphometric measurements (PND 60) were evaluated. There were no treatment-related findings on brain weights, gross and morphometric brain measurements in perfused rats, neuropathologic effects in the CNS or PNS, and no effects on brain myelin. In non-perfused rats (P1 adults, PND 22, PND 70, PND 139 F1 offspring), there were no adverse effects on brain weight and brain histopathology.

Developmental Immunotoxicity (DIT): Based on the SRBC antibody-forming cell assay (PND 66-70) and Natural Killer Cell assay (PND 87-93), there was no evidence of developmental immunotoxicity.

The parental systemic LOAEL is 800 ppm (40 mg/kg bw/day in males), based on

nephrotoxicity manifested as increased kidney weights, and degenerative lesions in the proximal convoluted tubules in the main study P1 rats. The parental systemic NOAEL is 300 ppm (15 mg/kg bw/day in males). No toxicologically relevant effects were identified in P1 females or in the GD 17 satellite female groups

The thyroid toxicity NOAEL is established at 800/600 ppm (40/30 mg/kg/day in males and females, respectively), the highest dose tested. The thyroid effects noted in the database were considered to be adaptive.

The offspring LOAEL is 300 ppm (15 mg/kg bw/day), based on kidney toxicity manifested as increased kidney weights and increased incidence of degeneration of the proximal convoluted tubules. The offspring NOAEL is 100 ppm (5 mg/kg bw/day).

The DNT offspring (PND 21-60) LOAEL is >800/600 ppm (40 mg/kg bw/day in males, 30 mg/kg bw/day in females), based on the lack of evidence of DNT (FOB parameters, motor activity, and acoustic startle response). The DNT offspring NOAEL is 800 ppm/600 ppm (40 mg/kg bw/day in males, 30 mg/kg bw/day in females), the highest dose tested.

The DIT offspring (PND 139) LOAEL is >800/600 ppm (40 mg/kg bw/day in males, 30 mg/kg bw/day in females), based on the lack of evidence of DIT [SRBC antibody-forming cell assay (PND 66-70) and Natural Killer Cell assay (PND 87-93)]. The DIT offspring NOAEL is 800/600 ppm (40 mg/kg bw/day in males, 30 mg/kg bw/day in females), the highest dose tested.

The reproductive LOAEL is > 800/600 ppm (40 mg/kg bw/day in males, 30 mg/kg bw/day in females), based on the lack of effect on estrous cyclicity, (P1 females, satellite GD 17 dams, Set 3 F1 offspring) or reproductive indices (mating, fertility, time to mating, gestation length, pre- and post-implantation loss, number of corpora lutea (satellite GD 17 dams), sperm parameters, ovarian follicle counts, and reproductive organ histopathology). The reproductive NOAEL is 800/600 ppm (40 mg/kg bw/day in males, 30 mg/kg bw/day in females), the highest dose tested.

This study is classified acceptable/non-guideline. The study does not satisfy a guideline requirement for 2, 4-D. It satisfies the data call-in requirements for 2, 4-D for OPPTS 870.3800 (Reproduction and Fertility Effects), OPPTS 870.6300 (Developmental Neurotoxicity), OPPTS 870.7800 (Immunotoxicity).

C. <u>STUDY DEFICIENCIES</u>: None that would adversely affect study interpretation.

APPENDIX A

Saghir, S. A., Zablotny, C. L. Marty, M. S. Perala, A. W. and Yano, B. L. (2008). A Dietary Dose Range-Finding and Pharmacokinetic Study of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in the Pregnant CRL:CD(SD) Rat and Its Offspring in Preparation for a Subsequent F1-Extended One-Generation Toxicity Study in Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Laboratory Project Study IDs 071153 and 071153A, May 2, 2008. MRID 47417901. Unpublished.

APPENDIX B

Saghir, S. A., Perala, A. W., and Clark, A. J. (2008). A dietary titration study of 2,4-dichlorophenoxyacetic acid (2,4-D) pharmacokinetics in female CRL:CD(SD) rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Laboratory Project Study ID 071210, April 29, 2008. MRID 47417902. Unpublished.

APPENDIX C

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Saghir, S. A., Mendrala, A. L., Bartels, M. J., Day, S. J., Hansen, S. C., Sushynski, J. M., and Bus, J. S. (2006). Strategies to assess systemic exposure of chemicals in subchronic/chronic diet and drinking water studies. *Toxicol. Appl. Pharmacol.* **211**, 245-260.